REMARKS

Claims 1, 2, 4-9, 13-27, 29-43, 45, and 46 were pending prior to this response. By the present communication, new claims 47-49 have been added. claims 1, 2, 4-9, 13-16, 18, 20-23, 26-28, 33, and 35-38 have been cancelled without prejudice and claims 17, 19, 24, 25, 29, 30, 31, 32, 34, 39, 40, 43 and 46 have been amended to define Applicants' invention with greater particularity. The amendments add no new matter, being fully supported by the Specification and originally filed claims. Support for new claim 49 is found on page 32 of the Specification. Accordingly, claims 17, 19, 24, 25, 29, 30-32, 34, 39-43, and 45-49 are currently pending.

The Attachments Allegedly Missing From the Previous Response

The Office Action alleges that there were no copies or attachments describing Applicants' post-filing studies present with the prior Response filed on September 23, 2004. However, the postcard mailed with the Response shows that the Response was 27 pages including attachments A-C. Thus, the references attached to the Response must have been mislaid by the PTO upon their receipt. For the convenience of the Examiner, Applicants again supply copies of the attachments allegedly missing from the prior Response and request the Examiner's consideration thereof in light of the argument in the prior and the present Responses.

The Rejection under 35 U.S.C. § 112, First Paragraph

Applicants respectfully traverse the rejection of claims 1, 2, 4-9, 13-27, 29-43 and 45-46 as allegedly lacking an enabling disclosure in the Specification. As claims 1, 2, 4-9, 13-16, 18, 20-23, 26-28, 33, and 35-38 have been cancelled without prejudice, the following remarks will pertain to the remaining claims as currently amended. All presently pending claims have been amended to pertain to injection of early attaching cells obtained from bone marrow that contain an adenoviral vector comprising a polynucleotide encoding one or more angiogenic factors selected from hypoxia inducing factor-1 (HIF-1), endothelial PAS domain protein 1 (EPAS1), Monocyte Chemoattractant Protein 1 (MCP-1), granulocyte-monocyte colony stimulatory factor (GM-CSF),

PR39, a fibroblast growth factor (FGF), and a nitric oxide synthase (NOS). The Examiner acknowledges that the specification is enabling for treatment of hind-limb ischemia in mice, but alleges that that the Specification does not reasonably provide enablement for promoting angiogenesis an "any tissue/organ of any animal or heart/limb tissue in a human. (Office Action, page 2).

According to the Office Action, the test for enablement is whether one skilled in the art can make use of the claimed invention coupled with information known in the art without undue experimentation (Office Action, page 2). The standard of undue experimentation is that set forth in In re Wands, including the following factors: 1) scope/breadth of claims; 2) nature of the invention; 3) state of the art and predictability; 4) amount of guidance provided; 5) number of working examples; and 6: amount of experimentation required (In re Wands, 8USPQ 2d 1400 (Fed. Cir. 1988). The factors will be addressed in the order they were presented in the Office Action.

Scope and breadth of claims. The Office Action states that the claims are broader than the scope of enablement because the composition claims are limited as "therapeutic" and are directed to transfected early attaching cells and the method claims are directed to enhancing collateral blood vessel formation in human "patients", thus involving "gene therapy." In Addition, the Examiner asserts that the ABMs are transfected with nucleic acids obtained from "any source" to express the named factors with the expectation of enhanced angiogenesis.

In response, to the Examiner's assertions, Applicants have amended the claims to clarify the claim scope. First, the term "therapeutic" has been deleted from the composition claims. In the method claims, the term "patient" has been deleted. In addition, the tissue recited is limited to "muscle tissue" suffering from restricted blood supply. Thus, neither the composition nor the method claims, as presently amended, recite language that can be construed to require a human subject.

Although the Examiner does not fully articulate the exact nature of concern regarding the unspecified "source" of the nucleic acids used to transfect the cells, it appears that the Examiner fears practice of the invention will cause an immune response to factors expressed from non-

autologous nucleic acids. However, the cytokines and other factors recited by the claims are transcription factors and other very small proteins, and/or proteins that are highly conserved across species for which the nucleic acid sequences were well known at the filing of the application, including those for murine and human factors. Those of skill in the art practicing the invention would have known to use a species-matched DNA for encoding the desired cytokine or factor. Moreover, the Examiner has provided no evidence that use of a non-homologous or non-species matched factor would either fail to be effective or that use of such small proteins and/or such proteins that are highly conserved across species, in vivo would generate an immune response if a non-homologous or non-species matched sequence were to be used.

Accordingly, Applicants submit that the scope and breadth of the currently amended claims is not too broad and that the claims can be practiced without undue experimentation.

Nature of the invention. The Office Action asserts that all claims are directed to treatment due to use of the term "patient" in the method claims and "therapeutic" in composition claims. Applicant disagrees that these terms identified by the Examiner necessarily require or refer to a human subject. However, for clarity the claims have been amended to delete any language that could be construed to require treatment of a human subject. Applicants respectfully submit that the invention composition comprising bone marrow-derived early attaching cells containing an adenovirus vector comprising polynucleotide(s) encoding at least one of the recited factors is intended to be can be used in animal model research and is presently being used by many experimenters, as is discussed in full detail below.

In addition, it should be noted that the Examiner does not allege that the Specification fails to teach those of skill in the art how to make the claimed composition or how to use it to enhance collateral blood vessel formation by injection into muscle tissue, as defined by amended claim 17. Applicants submit that the Specification contains Examples that describe the procedure used to make and use the claimed composition in such applications. Example 7, for instance, describes harvesting of bone marrow cells from pigs, culture of the cells in vitro to obtain the "early attaching cells," which are characterized as "mostly monocytes, endothelial precursor cells, or

other hemopoietic lineage cells" (¶ [0108]), in other words progenitor cells. Similarly, the procedure for transfection of the early attaching cells using an adenovirus vector is fully described (¶[0110]. In vivo use of early attaching cells that have been transfected with a polypeptide encoding HIF-1α in a mouse ischemic hind limb model showed that transduction of MSCs with HIF-1α was effective to "optimize the collateral-enchancing effects of a cell-based strategy for increasing collateral flow in ischemic tissue" (¶ [0125] when the cells were injected into muscle tissue suffering a loss of blood supply.

Thus, Applicants submit that the making and use of the invention composition in the invention methods, as defined by the currently amended claims, is described in the Specification in such a way as to enable those of skill in the art to make and use the invention without undue experimentation.

State of the art/Unpredictability of the art. The Office Action asserts that the Specification does not teach how to use the claimed methods and compositions therapeutically commensurate with the scope of the claims. In particular, the Examiner asserts that at the time the application was filed, gene therapy was a "highly unpredictable art with poor efficiency of delivery of the transgene to the target cells, poor transformation efficiency of target cells; unpredictable and transient expression of the transgene in target cells, etc." (Office Action, page 4).

At the outset it should be noted that all currently amended claims, both composition and method, require steps that distinguish the invention from the types of methodologies that form the basis of the articles cited by the Examiner as showing unpredictability in the art of "gene therapy". In the present invention, donor bone marrow progenitor cells are transfected ex vivo and the transfected *donor cells are used as a vector* to deliver the transgene directly into muscle by injection. Thus, the vector itself, which contains the transgene, is not directly administered into the circulation or muscle tissue, but is injected into the bone marrow-derived cell ex vivo, which method shields the vector from detection by the immune system of the recipient, considerably

lessening, if not completely eliminating, the type of recipient immune response to the viral vector described by the St. George reference of record herein as cited by the Examiner.

Alternatively, in the invention methods the transfected donor cells are further prepared by ex vivo culture to obtain conditioned medium containing not only the usual products secreted by the cells but also the protein product of the transgene and/or the protein products derived from the effects of the transfected gene product on the transduction pathways of the transfected cell. The transgene expression products in the conditioned medium will be obtained ex vivo, *prior to injection of the conditioned medium* into muscle tissue, such as heart or limb tissue. In particular, it should be noted that in the case wherein the composition injected contains the conditioned medium, the transgene is required to have been expressed before the invention composition is injected into the muscle tissue.

Further in support of the rejection for lack of enablement, the Examiner states: "More particularly, with respect to gene therapy, the transduced cells in a human patient would need to express the angiogenic factor for a threshold period of time to promote angiogenesis..." (Office Action, page 5) However, in studies conducted by the inventors and others, it has been shown that adenoviral gene expression lasts for at least weeks, and perhaps months. The application teaches that cellular survival of the transgene and gene product expression in situ following injection of transduced cells into adductor muscle of mice showed in situ expression of the transgene was maintained through day 14. The time table for increasing collateral flow in ischemic muscle tissue is much briefer. Applicants have also demonstrated in data published using normal dogs that administration of FGF-2 to ischemic dog hearts for only 2 days increases collateral flow. The Examiner has provided no reason to believe that a substantially different result would be obtained in humans. Applicants and others have shown in published studies that development of collateral blood flow in muscle tissue upon injection of the transduced blood marrow progenitor cells takes place rapidly following onset of ischemia (as quickly as 3 days after ischemia onset). Thus, there is no support for the Examiner's inference that the transgene would not be expressed for a sufficient time to effect enhanced development of collateral blood vessels in the muscle tissue into which it is directly injected.

Thus, Applicants submit that the Examiner's concerns regarding supposed lack of efficiency of transfection and unpredictable and transient expression of the transgene in target cells are unfounded.

While the Examiner's assertions may be valid as applied to conventional routes of gene therapy, Applicants have shown that at least in the case at issue of early attaching cells obtained from bone marrow, these cells are transfected with a heterologous gene ex vivo at efficiency of "over 90%" (Specification, ¶ [0117]). High transfection efficiency in human progenitor cells has also been shown (See attached Exhibit A, T Watanabe et al., Blood (1996) Jun 15; 87(12):5032-9).

The Examiner's assertion that deleterious viral proteins will be produced in the conditioned medium resulting from use of an adenovirus vector" (Office Action, page 4) is equally unfounded. Adenoviral vectors, as the term is used in the art, are not intact adenoviruses, but are routinely constructed so that essentially no viral genes are transcribed. The only gene transcribed is the transgene that has been inserted into the vector. For example, the Specification describes such "replication-deficient adenoviral vectors" in ¶ [0050-51]. The actual adenoviral HIF-1α/VP16 vector used to transfect MSCs in Example 7 of the Specification is covered by U.S. Patent No. 6,093,567 and was used in collaboration with Genzyme Corporation, owners of the '567 patent. The same construct is presently being injected directly (without ex vivo transfection of bone marrow progenitor cells) into *human* heart and limb muscle in FDA approved clinical trials being conducted by Genzyme Corporation to show efficacy for angiogenesis. Thus, it appears that the FDA believes that the use of adenoviral vectors is sufficiently safe and effective for use in humans as well as in other mammals.

The St. George reference actually supports these statements with regard to local administration of adenoviral vectors as follows:

> The January 2002 issue of Human Gene Therapy contains two studies describing the responses in nonhuman primates. Studies in non-human primates may be the most instructive to our understanding of Ad-mediated toxicity as the responses have been shown to mirror those described in patients. The findings of

> studies conducted in primates, including humans, are summarized briefly in Table 1. It is important to note that on balance, other manuscripts in that issue of Human Gene Therapy concluded that in studies using several routes of local administration of low to intermediate doses of Ad vectors were well-tolerated in humans.

(St. George, page 1137, first col.) Thus, the art has shown that adenoviral vectors are on balance safe and effective in humans as in primates.

Applicants respectfully disagree as well with the Examiner's assertion that insertional mutagenesis is a dreaded potential complication of adenoviral gene delivery, including the development of leukemia. It is well known in the art that *retrovirus* can insert into the genome and thereby can cause insertional mutagenesis. Retrovirus have recently been shown capable of causing leukemia. However, it is also very well known in the art that adenovirus vectors of the type described in the Specification do not insert into the genome. Adenoviruses always reside in an extrachromosomal location. Applicants submit that adenoviruses have never been found to cause insertional mutagenesis and never have been associated with cancers, whether injected directly into muscle or not.

The one recent adverse incident involving an adenoviral vector when used for human gene therapy (in a clinical trial conducted by the University of Pennsylvania) should be distinguished from the present invention. The University of Pennsylvania clinical trial involved a case wherein the adenoviral vector was injected in very high concentrations (10^{13}) directly into the artery supplying the patient's liver. The adenoviral vector was not introduced into a muscle or into the blood stream via the patient's own progenitor cells that had been transfected ex vivo with the adenoviral vector.

Applicants have shown in Example 6-8 of the Specification that bone marrow-derived early attaching cells, such as MSCs, can be transfected with an adenovirus vector with high efficiency and that conditioned medium alone obtained by growing such transfected cells contains numerous angiogeneic cytokines and exerts biologic effects that are compatible with the capacity to develop collaterals. Thus, Applicants studies have shown a very high transfection efficiency of the early attaching cells and transient transgene expression of sufficient duration to accomplish the claimed result of enhanced collateral development of blood vessels in the injected muscle tissue.

This study as well as the arguments above effectively traverse the Examiner's arguments that use of an adenoviral vector to transfect cells used in cell therapy is unpredictable and unreliable.

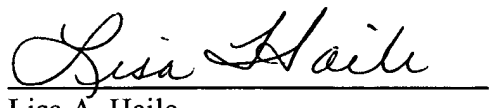
Amount of guidance provided/Number of working examples/Amount of experimentation required. The Examiner's assertions with regard to all three of these Wands categories can be summed up in the following statement: "...no significant relevant guidance is provided with respect to gene therapy or transfection of ABMs and treatment of a human patient" (Office Action, page 7). However, the currently amended claims do not recite any language that requires or implies a human patient. Moreover, Applicants submit that the studies described in Example 6-8 of the Specification are sufficient to guide one of ordinary skill in the art to perform the claimed invention in subjects without undue experimentation, as shown by published studies performed by others since the publication of the present invention. For example, Exhibit B attached (E. C. Perin et al., *Circulation* (2003) 107:r75-r83) describes transendocardial injection of mononuclear cells obtained from autologous bone marrow cells into patients with end stage ischemic heart disease safely promoted neovascularization and improved perfusion and myocardial contractility. This study, which discusses the similarity of outcomes in animal and human studies, shows that even without transfection of the cells with an adenoviral vector expressing an angiogenic factor, humans react to intracardiac injection of bone marrow-derived progenitor cells in the same way that Applicants illustrated in the mouse hind-limb study. A similar therapeutic result is described in attached Exhibit C (M.B. Britten, et al., *Circulation* (2003) 108:2212-2218), resulting from infusion of bone marrow-derived progenitor cells into infarcted myocardial artery of human subjects. MRI was used to show significant improvement in global LV EF and reduced end-systolic volumes using this method. Yet another study in humans is described in attached Exhibit D (Extract of K.C. Wollert et al., *Lancet* (2004) 364(9429):121-2), which describes tests in 60 patients in which intracoronary transfer of autologous bone marrow cells promoted improvement of left-ventricular systolic function in patients after acute myocardial infarction.

Thus, although the level of skill in the art required to perform the claimed invention is high, Applicants respectfully submit that there are sufficient examples and guidance in the specification for those of skill in the art to practice the invention as defined by the current claims without undue experimentation. Accordingly, reconsideration and withdrawal of the rejection for alleged lack of enablement are respectfully requested.

In view of the above amendments and remarks, Applicants request favorable action on all pending claims. If the Examiner would like to discuss any of the issues raised in the Office Action, the Examiner is encouraged to call June Learn at (702) 614-7219 so that a prompt disposition of this application can be achieved.

Respectfully submitted,

Date: March 15, 2005



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Attachments:
1. Al-Khaldi et al., "Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model", *Ann Thorac Surg.* 2003 Jan; 75(1):204-9
2. Fuchs et al., "Catheter-based autologous bone marrow myocardial injection in no-option patients with advanced coronary artery disease: a feasibility study", *J Am Coll Cardiol.* 2003 May; 41(10):1721-4
3. Kinnaird et al, "Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms", *Circ Res.* 2004 Mar 19;94(5):678-85
4. Kinnaird et al., "Local Delivery of Marrow-Derived Stromal Cells Augments Collateral Perfusion through Paracrine Mechanisms", *Circulation* (2004) Mar 30;109(12):1543-9

Exhibit A — Abstract T Watanabe et al., Gene transfer into human bone marrow hematopoietic cells mediated by adenovirus vectors, *Blood* (1996) Jun 15; 87(12):5032-9)

Exhibit B — E. C. Perin et al., Transendocardial, Autologous Bone Marrow Cell Transplantation for Severe, Chronic Ischemic Heart Failure, *Circulation* (2003) 107:r75-r83

Exhibit C — M.B. Britten, et al., Infarct Remodeling After Intracoronary Progenitor Cell Treatment in Patients with Acute Myocardial Infarction, *Circulation* (2003) 108:2212-2218

Exhibit D — Abstract of K.C. Wollert et al., Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomized controlled clinical trial, *Lancet* (2004) 364(9429):121-2

CARDIOVASCULAR

Therapeutic Angiogenesis Using Autologous Bone Marrow Stromal Cells: Improved Blood Flow in a Chronic Limb Ischemia Model

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Background. We evaluated the effect of autologous marrow stromal cells (MSCs) on neovascularization and blood flow in an animal model of chronic limb ischemia.

Methods. Chronic hind limb ischemia was created by ligating the left common iliac artery of male Lewis rats. Three weeks after ligation, 5.0 million LacZ$^+$MSCs (n = 10) or culture medium (n = 10) were injected into the anteromedial muscle compartment of the left thigh. At 4 and 6 weeks after injection, half the animals (n = 5) from each group underwent femoral artery ultrasonic blood flow measurements of the ischemic and nonischemic limbs to obtain a flow ratio. The animals also underwent angiography and measurements of blood vessel density and arteriolar density. Qualitative histologic assessment of the limb muscles was performed.

Results. LacZ$^+$MSCs were found to differentiate into endothelium (F VIII$^+$), vascular smooth muscle (positive α-smooth muscle actin), skeletal muscle (positive desmin), and adipocytes. Ischemic hind limbs where MSCs were implanted had greater vascular density and arteriolar density than control limbs ($p < 0.001$). Femoral artery flow index (left femoral artery flow/right femoral artery flow) was 0.89 ± 0.12 and 0.90 ± 0.06 for rats injected with MSCs measured at 4- and 6-weeks, respectively, compared with 0.50 ± 0.15 and 0.50 ± 0.10 for the control rats ($p < 0.001$). Angiography demonstrated reconstitution of the left femoral artery in rats that received MSC implantation through pelvic and abdominal wall collateral formation.

Conclusions. Local MSC implantation induces a neovascular response resulting in a significant increase in blood flow to the ischemic limb. Marrow stromal cells are also capable of spontaneously regenerating the various components of muscular tissues.

(Ann Thorac Surg 2003;75:204–9)

Marrow stromal cells (MSCs) are pluripotent adult-type stem cells that have been shown to differentiate into different cellular phenotypes, including osteocytes, chondrocytes, and cardiomyocytes [1, 2]. Several studies have suggested that these mesenchymal stem cells undergo milieu-dependent differentiation, and as such have the potential to participate in organ repair and regeneration [3, 4]. Previous studies from our laboratory have demonstrated that these cells have the potential to induce a neovascular response in murine matrigel angiogenesis assay [5]. In the present study, we explored the ability of autologous MSCs to not only induce new blood vessel growth but also improve blood flow in an animal model of chronic limb ischemia.

Presented at the Poster Session of the Thirty-eighth Annual Meeting of The Society of Thoracic Surgeons, Fort Lauderdale, FL, Jan 28–30, 2002.

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Material and Methods

Animals

Male Lewis rats (250 to 275 g) were obtained from Charles River Laboratories (Laprairie Co, Quebec, Canada). These isogenic rats were used as donors and recipients of MSCs to simulate autologous implantation. All animals were studied using guidelines published in "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication 85-23, 7th edition, revised 1996).

Harvest and Culture Expansion of Bone Marrow Stromal Cells

We sacrificed male Lewis rat and harvested bone marrow by flushing femurs and tibias with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 U/mL penicillin/streptomycin. We plated whole marrow in tissue culture dishes and 5 to 7 days later discarded the nonadherent hematopoietic cells and maintained the adherent bone marrow stromal cells at 37°C with 5% CO_2.

Generation of LacZ$^+$ Marrow Stromal Cells

Marrow stromal cells were labeled with retroviral particles carrying the LacZ gene that codes for the prokaryotic

β-galactosidase enzyme. The retroviral particles were derived from GP+AM12-nlsLacZ cell line. These cells produce 1.6×10^5 infectious particles/mL as determined by titer assay on NIH 3T3 cells. Marrow stromal cells transduction was performed twice per day for 3 consecutive days (with 6 μg/mL lipofectamine). Following LacZ transduction, 85% to 90% of all MSCs expressed detectable β-galactosidase activity as assessed by X-gal staining, which served as a cellular marker for the presence of MSCs.

Creation of Hind Limb Ischemia and Marrow Stromal Cell Implantation

Thirty minutes before anesthesia, each rat received the narcotic analgesic buprenorphine (Temgesic, 0.01 mg/kg subcutaneously, Reckitt and Colman Pharmaceuticals Inc, Wayne, NJ). Anesthesia was induced by 4% isoflurane in gas (30% O_2 and 70% air). After induction the rat was transferred to a servo-controlled, heated table and body temperature was maintained at 37°C. The isoflurane was reduced to 2% delivered by a facemask. Using sterile technique, the abdomen was opened through a lower midline incision, the aortic bifurcation was identified and the left common iliac artery was ligated using 5-0 silk at its midpoint as described by Rochester and coworkers [6]. Anesthesia was discontinued and animals were allowed to recover completely before replacing them in their cages. No mortality, paralysis, gangrene, or ulcers occurred in any group. Three weeks after ligation, 5.0 million $LacZ^+$ MSCs (MSC group, n = 10) or culture medium (control group, n = 10) were injected into the anteromedial muscle compartment of the left thigh.

Experimental Protocol for Angiogenesis Assessment

At 4 and 6 weeks after the creation of hind limb ischemia, 5 rats from the treatment group and 5 rats from the control group were placed under isoflurane anesthesia as described above with body temperature maintained at 37°C on a servo-controlled, heated table. The trachea was intubated followed by ventilation using Harvard rodent ventilator (Harvard Apparatus Co, Inc, South Natick, MA) delivering 3 mL tidal volume at 70 breaths per minute. A cannula (PE50) was inserted into the right femoral vein. Constant infusion delivered 1% of body weight per hour, which contained 2% bovine serum albumin in normal saline. During the 1-hour postsurgical equilibration period, inspired anesthetic concentration was titrated to the minimum concentration that precluded a response when the tail was pinched. The following measurements and tests were performed:

BLOOD FLOW MEASUREMENT. Blood flow was measured in both femoral arteries using Transonic Systems Inc (Ithaca, NY) T106 transient time ultrasound flowmeter (R1 probe). The flow probe was placed around the proximal part of the femoral artery and blood flow measurement was taken after a 1-hour equilibration period.

HIND LIMB ANGIOGRAPHY. Under isoflurane anesthesia, the abdomen was opened and a cannula was inserted into the abdominal aorta with the tip below the renal arteries. The cannula was used to infuse 0.5 mL of Hypaque-M 60% contrast media (Nycomed Image AS) in 2 seconds. Images were taken using OEC 9600 mobile C-arm equipped with a 12-inch image intensifier and digital subtraction angiography capabilities. Images were acquired at a frame rate of 4 frames/sec with image magnification set at maximum.

MUSCLE SPECIMENS RETRIEVAL AND PROCESSING. After blood flow measurements and angiography, rats were killed with pentobarbital overdose. The rat systemic circulation was flushed through the left ventricle with 20 mL of cold (4°C) phosphate-buffered solution followed by 20 mL of cold 2% paraformaldehyde. Muscle specimens obtained from both hind limbs were cut into thin slices and placed in 2% paraformaldehyde at 4°C for 24 hours followed by incubation in X-gal staining solution, which consisted of 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6 \cdot 3H_2O$, 0.01% sodium deoxycholate, 2 mmol/L $MgCl_2$, 1 mmol/L EGTA, and 1 mg/mL X-gal made in wash solution (phosphate-buffered saline with 0.02% NP40). The X-gal solution was adjusted to pH of 8.0 for maximum specificity of the staining as described before [7]. After 16 hours, we fixed the specimens in 10% formalin and embedded them in paraffin. Sections were cut at 3 to 4 μm.

IMMUNOHISTOCHEMICAL STAINING. Random sections from each specimen were deparaffinized and standard three-steps immunohistochemical staining was performed using the following primary antibodies (on separate sections): polyclonal rabbit anti-human factor VIII, monoclonal mouse antihuman desmin (both from DAKO Corp, Carpinteria, CA), and monoclonal mouse antihuman α-smooth muscle actin (Sigma, St. Louis, MO). All antibodies are known to cross-react with the corresponding rat antigens. Universal biotinylated antimouse and rabbit secondary antibody (Ventana Medical Systems, Tucson, AZ) was used followed by avidin-peroxidase complex binding. The reaction was developed with diamino benzidine substrate.

MICROSCOPY AND VASCULAR DENSITY. All sections were examined with an Olympus BX60 microscope. Digital images were transferred to a computer equipped with Image Pro software (Media Cybernetics). In H&E-stained sections, we considered as blood vessels only tubular structures with patent lumen that were lined with endothelium. In sections stained with antifactor VIII antibody, we considered as blood vessel only tubular structures that were factor VIII+. For vascular density measurements using sections stained with antifactor VIII, we counted the number of blood vessels in 10 random high power fields (magnification ×400) selected using the systemic sampling with random start technique. We calculated the average of the 10 high power fields (hpf) and expressed the vascular density as blood vessels/hpf. Arterioles were identified by the presence of smooth muscles in their wall in sections stained with anti-α-smooth muscle actin and were counted in the same technique.

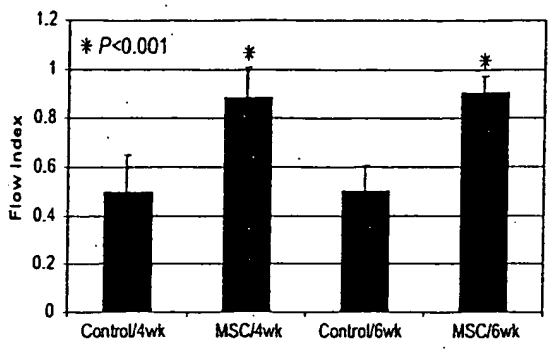


Fig 1. Comparison of femoral artery flow index in the treatment (marrow stromal cell [MSC]) and control subgroups at 4 and 6 weeks after intramuscular injection.

Statistical Analysis

All data are expressed as the mean ± SD. Statistical analysis was carried using the SPSS version 10.0 (SPSS Inc.). A p-value of less than 0.05 was considered to be statistically significant. Analysis of variance was used to compare the means of the different groups of animals followed by Bonferroni multiple comparison test.

Results

Changes in Femoral Artery Flow

To account for animal-to-animal and minute-to-minute variation in hemodynamics, we used the femoral artery flow index (FAFI) to standardize the femoral artery flow measurements and allow for comparisons between different animals.

FAFI = Left femoral artery flow/Right femoral artery flow.

FAFI has no units and equals 1 in normal rats in which the left common iliac artery was not ligated (data are not shown). Marrow stromal cell implantation resulted in significant increase in the femoral artery flow index (Fig 1). FAFI was 0.89 ± 0.12 and 0.90 ± 0.06 for rats injected with MSCs measured at 4 and 6 weeks, respectively, compared with 0.50 ± 0.15 and 0.50 ± 0.10 for the control rats ($p < 0.001$).

Angiography

Hind limb angiography demonstrated complete occlusion of the left common iliac artery at the site of ligation in both the treatment and control groups (Fig 2). Angiography also showed absence of flow in the left femoral arterial system in the control rats. In rats that received MSCs implantation, there was reconstitution of the left femoral artery by collateral vessels derived from the abdominal wall and the contralateral pelvic arteries.

Vascular Density

About a fourfold increase in vascular density was found in the left hind limb muscle specimens of rats that received MSCs implantation (Fig 3). Vascular density in left limb muscles was 4.5 ± 2.0 and 5.4 ± 0.7 for rats injected with MSCs and measured at 4 and 6 weeks, respectively, compared with 1.2 ± 0.6 and 1.1 ± 0.3 for control rats.

Arteriolar Density

Marrow stromal cell implantation was associated with increased arteriolar density (about threefold) in the treated ischemic hind limbs (Fig 4). The arteriolar density in the left limb muscles was 1.6 ± 0.4 and 1.7 ± 0.5 for rats injected with MSCs and measured at 4 and 6 weeks, respectively, compared with 0.5 ± 0.1 and 0.4 ± 0.2 for control rats.

In Vivo Differentiation of Marrow Stromal Cells and Vasculogenesis

LacZ$^+$MSCs identified by their indigo blue color when stained with X-gal were found to undergo in vivo phenotypic differentiation into endothelial cells that participate in the formation of new blood vessels (Fig 5). These LacZ$^+$MSCs express the endothelial markers factor VIII (Fig 6A, 6B) and CD34 (data not shown). Both markers are not expressed by MSCs before implantation, as found by immunohistochemical and fluorescent-activated cell sorting analysis (data not shown). LacZ$^+$MSCs also differentiate into vascular smooth muscles and express α smooth muscle actin (Fig 6C, 6D). In vivo differentiation into other components of muscular tissue was noted, including skeletal muscle fibers (Fig 6E) with expression of desmin (Fig 6F) and adipocytes (Fig 6G).

Comment

Autologous MSCs are desirable for cell therapy as they are abundant, easy to harvest, and easy to culture expand to achieve sufficient numbers for therapeutic purposes. They are also relatively easy to modify genetically. Although several studies have demonstrated the potential of MSCs to differentiate into different cell types [1, 2], from a therapeutic point of view functional improvement

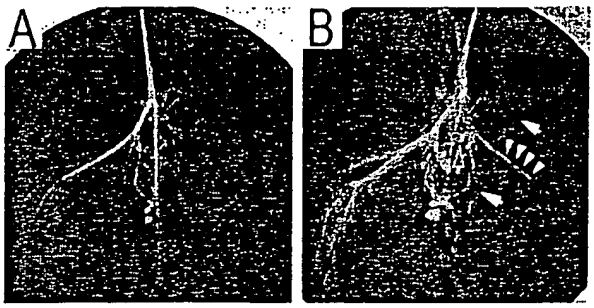
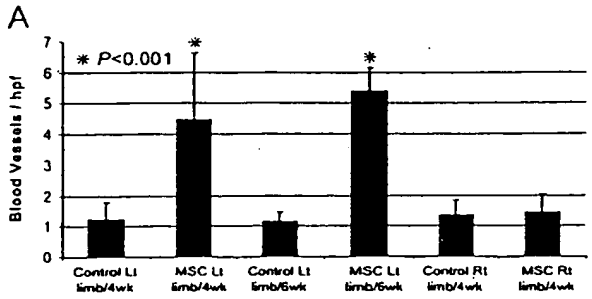


Fig 2. Angiograms of the pelvic and hind limb arteries 4 weeks after intervention. (A) Control rat angiogram showing no flow of contrast distal to the site of left common iliac artery ligation (arrow). (B) Angiogram of rat that received marrow stromal cell implantation showing the site of left common iliac artery ligation (black arrow). Reconstitution of the left femoral artery (arrowheads) by pelvic and abdominal wall collaterals (white arrows) is visible.



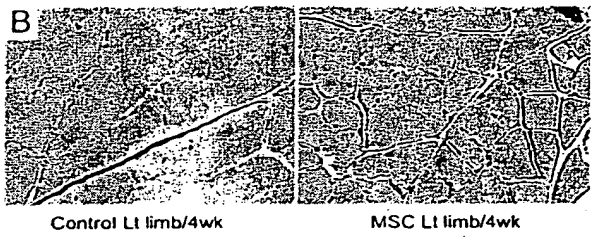


Fig 3. (A) Comparison of the vascular densities in muscle specimens obtained from either left or right limbs in the treatment (marrow stromal cell [MSC]) or control group at 4 and 6 weeks after intervention. (hpf = high power field; Lt = left; Rt = right.) (B) Immunohistochemical staining for the endothelial marker factor VIII in muscle specimens from ischemic left hind limbs clearly demonstrates marked increase in blood vessels in ischemic muscle tissues where MSCs were implanted compared with controls. (Magnification ×100 before 46% reduction.)

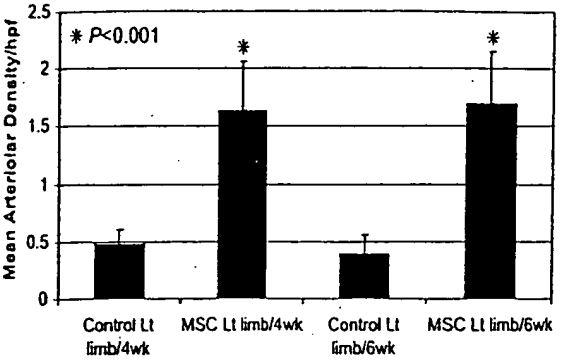


Fig 4. Comparison of the arteriolar densities in muscle specimens obtained from either left or right limbs in the treatment (marrow stromal cell [MSC]) or control group at 4 and 6 weeks after intervention. Arterioles were identified as blood vessels with smooth muscle in their walls as demonstrated by immunohistochemical staining with anti-α smooth muscle actin. (hpf = high power field; Lt = left.)

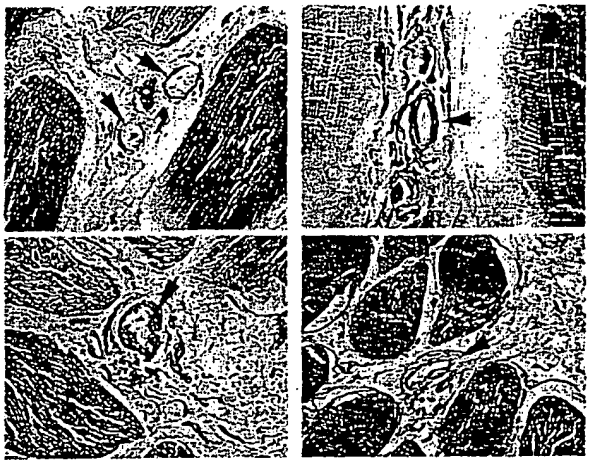


Fig 5. Histologic sections of muscle tissues where marrow stromal cells (MSCs) were implanted stained with X-gal to identify the LacZ⁺ MSCs and counterstained with eosin. Arrows point to several blood vessels where LacZ⁺ MSCs (indigo-blue color) are actively participating in the formation of new blood vessels (ie, vasculogenesis) with histologic features suggestive of endothelial differentiation. (Magnification ×200 before 43% reduction.)

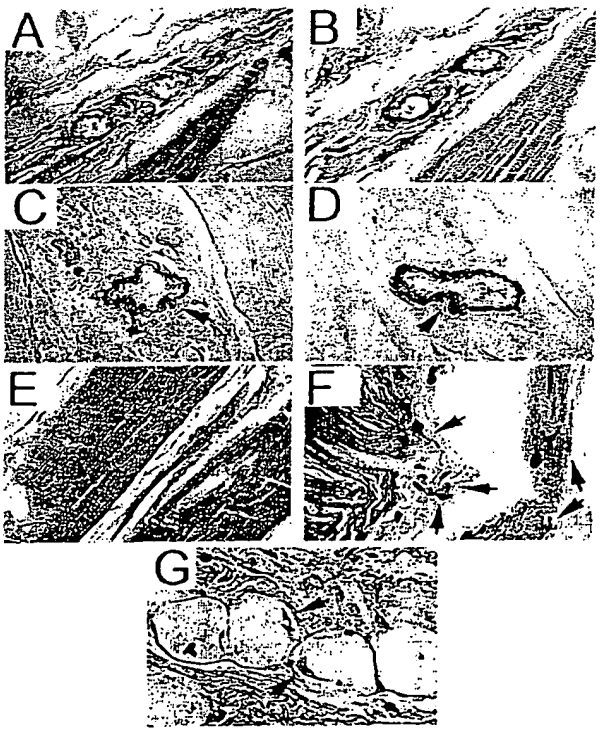


Fig 6. Histologic sections of muscle tissues in which marrow stromal cells (MSCs) were implanted. (A) X-gal- and eosin-stained section showing LacZ⁺ MSCs forming new blood vessels and express factor VIII as shown in (B), which is a consecutive section that was stained with X-gal and anti-factor VIII antibodies. (Magnification ×200 before 35% reduction). (C) and (D) sections stained with X-gal and anti-α smooth muscle actin that show LacZ⁺ MSCs (arrows) differentiating into vascular smooth muscle and expressing α smooth muscle actin. (Magnification ×400 before 35% reduction.) (E) X-gal- and eosin-stained section showing LacZ⁺ MSCs differentiating into skeletal muscle fibers. (Magnification ×200 before 35% reduction.) (F) Section stained with X-gal and antidesmin showing LacZ⁺ MSCs (arrows) expressing desmin. (Magnification ×200 before 35% reduction.) (G) X-gal- and eosin-stained section showing LacZ⁺ MSCs (arrows) differentiating into adipocytes. (Magnification ×400 before 35% reduction.)

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is important. In this study, we hypothesized and demonstrated that MSCs not only induce a neovascular response when placed in ischemic tissue, but also improve blood flow to that tissue.

We implanted MSC locally in a rat hind limb ischemia model as described by Rochester and colleagues [6] as opposed to a coronary ischemic model for a number of reasons. The purpose of the study was to demonstrate a proof of principle, namely that MSCs can improve blood flow to an ischemic organ. Whether the ischemia was myocardial, cerebral, or limb was not crucial for this study. What was crucial was that the model be simple. Indeed, we found that the hind limb ischemia model of the rat is easy to perform, is associated with a low procedural mortality, results in a stable and predictable reduction in blood flow (around 50% of normal), and allows for an easy method for the determination of blood flow using ultrasound of the femoral artery. Larger animal models are generally needed for coronary ischemia and are associated with higher costs and increased mortality; they also require a more elaborate method of evaluating blood flow such as radioactive or colored microspheres [8]. One should not assume, however, that the improved blood flow after MSC implantation found in this study can be generalized and assumed to occur in the rat coronary circulation or, more importantly, in the human situation and this is clearly a limitation of the study. Our findings do at least support the concept that MSCs can be used to improve blood flow in ischemic tissue.

Our flow data demonstrate that MSC-related neovascularization produces a physiologically significant increase in the femoral artery blood flow index from a base line of 50% in controls to 90% in the treated limb. The mechanisms by which local implantation of MSCs improve blood flow are probably multifactorial, as the neovascularization which we observed was of three types: angiogenesis, vasculogenesis, and collateral vessel formation.

Angiogenesis

Host-derived vascular density increase significantly in the treated hind limb as compared with control limbs. The local angiogenic effect due to MSC implantation may occur in part due to MSC-mediated vascular endothelial growth factor (VEGF) production. We have previously shown in a matrigel model [5] that the host-derived angiogenic response following MSC implantation can be reduced by 80% by blocking VEGF receptors using monoclonal antibodies, suggesting an important role for VEGF. In addition, in unpublished data, MSCs exposed to hypoxic conditions upregulate VEGF-mRNA expression and hypoxic inducible factor. Other growth factors may be upregulated (fibroblastic growth factor or transforming growth factor) but await further study and evaluation. The impact of an increase in local small vessel vascular density to overall blood flow is, however, probably minor [9]. Nevertheless, the increased density may act by reducing the overall resistance to flow through an increase in total vascular cross-sectional area.

Vasculogenesis

Marrow stromal cells appeared to participate in forming arteriole-like vessels and were found to express endothelial markers when lining the vessel wall lumen and express smooth muscle markers when incorporated into the vessel wall. This finding is not surprising because MSCs are pluripotent cells and have been shown previously to undergo differentiation into many cell types including adipose, chondrocytes, and myocardial cells. In a previous study we have shown that MSCs can undergo phenotypic change and express endothelial markers such as CD31 and VEGF receptor [5]. Therefore, in addition to stimulating a neovascular response, some cells actually appear to participate and become incorporated into the new blood vessel formation as well as surrounding adipose and skeletal muscle. This observation is consistent with those of others who describe this phenomenon as "milieu-dependent" differentiation [1]. The role of these newly created arteriolar-like structures to blood flow is not entirely known, but arteriogenesis may allow for sufficiently large vessels to improve local blood flow if they also communicate with an inflow source of blood [9].

Collateral Vessel Formation

Improved collateral vessel formation in the treated group was evident by angiography and occurred from the abdominal wall and contralateral pelvis and this improvement appears to be directly responsible for the improved blood flow to the treated limb. How these collaterals develop from an area that is remote from the local effects of MSC- mediated angiogenesis is not entirely clear. Normally, collateral formation that occurs following arterial occlusion, as in arteriosclerosis, is due to the recruitment of naturally preexisting small vessels that increase in size mainly due to a local increase in shear stress with resulting invasion of monocytes, cytokines, and growth factors (fibroblast growth factors and to a lesser extent VEGF); tissue ischemia per se is not thought to be an important stimulus for collateral formation [10]. In addition, the normal collateral formation which occurs after vessel occlusion is not always visible on an angiogram. The explanation for the robust collateral formation demonstrated in this model maybe due to one or a combination of the following: (1) The improved flow to the ischemic limb correlates with the local finding of an increase in vascular density of the ischemic treated limb suggesting that an increase in total vascular cross-sectional area might reduce the local resistance to flow thereby improving collateral formation. (2) Local injection of MSCs may act as a potent signal "sink" due to the increased secretion of growth factors as previously described as well as other unidentified agents, which together stimulate collateral formation. (3) The local implantation of MSCs may have an as-yet unidentified systemic effect such as vasodilatation (perhaps VEGF mediated), which would promote collateral blood flow. Clearly, future studies are needed to elucidate the mechanisms involved to explain the collateral formation that occurs after local injection of MSCs.

We propose the use of autologous MSC implantation for potential use as cellular therapy for tissue ischemia. The ability of MSCs to promote collateral formation and actively participate in blood vessel formation and surrounding tissue repair suggests that they may have multiple therapeutic benefits in treating patients with limb or myocardial ischemia. The biological mechanisms underlying these observations need further research to identify the appropriateness and safety of MSC as cellular therapy in anticipated human treatment.

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Catheter-Based Autologous Bone Marrow Myocardial Injection in No-Option Patients With Advanced Coronary Artery Disease

A Feasibility Study

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OBJECTIVES We conducted a pilot study to evaluate the feasibility of transendocardial delivery of autologous bone marrow (ABM) strategy in patients with severe symptomatic chronic myocardial ischemia not amenable to conventional revascularization.

BACKGROUND Transendocardial injection of ABM cells appears to enhance perfusion of ischemic porcine myocardium.

METHODS Ten patients underwent transendocardial injection of freshly aspirated and filtered unfractionated ABM using left ventricular electromechanical guidance. Twelve injections of 0.2 ml each were successfully delivered into ischemic noninfarcted myocardium pre-identified by single-photon emission computed tomography perfusion imaging.

RESULTS Autologous bone marrow injection was successful in all patients and was associated with no serious adverse effects; in particular, there was no arrhythmia, evidence of infection, myocardial inflammation, or increased scar formation. Two patients were readmitted for recurrent chest pain. At three months, Canadian Cardiovascular Society angina score significantly improved (3.1 ± 0.3 vs. 2.0 ± 0.94, $p = 0.001$), as well as stress-induced ischemia occurring within the injected territories (2.1 ± 0.8 vs. 1.6 ± 0.8, $p < 0.001$). Treadmill exercise duration, available in nine patients, increased, but the change was not significant (391 ± 155 vs. 485 ± 198, $p = 0.11$).

CONCLUSIONS This study provides preliminary clinical data indicating feasibility of catheter-based transendocardial delivery of ABM to ischemic myocardium. (J Am Coll Cardiol 2003;41:1721–4)

Despite multiple clinical studies, efficacy of various angiogenesis strategies to improve myocardial perfusion remains unproven (1,2). Previous efforts, however, involved delivery of a *single* angiogenesis factor. Given the complexity of natural angiogenesis processes, such a strategy might provide a suboptimal stimulus to collateral development. Therefore, we tested a cell-based strategy, based on the hypothesis that the cells secrete, in a time- and concentration-appropriate manner, *multiple* angiogenesis factors needed for optimal collateral development.

Preclinical studies demonstrated that administration of autologous bone marrow (ABM)-derived cells to ischemic porcine myocardium is safe and appears to improve collateral flow (3,4). The present phase I pilot study was designed to examine the feasibility of percutaneous transendocardial injection of unfractionated ABM in patients with advanced coronary artery disease.

METHODS

Patient selection. Patients were eligible for the study if they had Canadian Cardiovascular Society (CCS) angina class III to IV despite best tolerated medical therapy, no conventional revascularization treatment option, reversible myocardial ischemia on single-photon emission computed tomography imaging, and at least one major epicardial vascular conduit with >70% and one with <70% diameter stenosis. Exclusion criteria included abnormal hemoglobin, platelets, or leukocyte count; conditions that may adversely affect bone marrow (BM) (such as malignancy or human immunodeficiency virus infection), ejection fraction (EF) <30%, and recent (<1 month) myocardial infarction or class III unstable angina. The study was approved by the local Institutional Review Board of each of the sites and the U.S. Food and Drug Administration. Informed consent was obtained from all patients before enrollment.

BM. Bone marrow was aspirated from the iliac crest, mixed with heparin (20 U per ml of BM), and filtered as previously

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Abbreviations and Acronyms

ABM	= autologous bone marrow
BM	= bone marrow
CCS	= Canadian Cardiovascular Society
CK	= creatine kinase
EF	= ejection fraction
HUVECs	= human umbilical endothelial cells
LV	= left ventricle/ventricular
MCP-1	= macrophage chemoattractant protein-1
SPECT	= single-photon emission computed tomography
VEGF	= vascular endothelial growth factor

described (4). Filtered BM was assessed morphologically and tested for viability (TriPan Blue staining), absence of clots, bone spicules, and gross bacterial contamination.

Bone marrow cell population was analyzed by fluorescence-activated cell sorting using anti-CD34, anti-CD45, anti-CD117, anti-CD3, anti-CD4, anti-CD8, and anti-CD41 antibodies (Becton Dickinson, Burlingame, California).

Assessment of BM-derived angiogenic growth factors. Freshly aspirated and filtered BM cells of nine patients were cultured for four weeks, and levels of vascular endothelial growth factor (VEGF) and macrophage chemoattractant protein-1 (MCP-1) in the conditioned medium were measured separately for each of the samples as previously described (4). The effect of conditioned medium on human umbilical endothelial cell (HUVEC) proliferation was also measured by direct counting (4).

BM injection. Left ventricular (LV) electromechanical mapping and injection procedures (Biosense, Johnson and Johnson, Warren, New Jersey) have been described (5,6). Upon completion of LV mapping, 12 injections of 0.2 ml of BM each (total 2.4 ml) were delivered into a predefined myocardial ischemic territory (SPECT data) approximately 1 cm apart. We avoided injecting into the LV apex or mitral valve area (Fig. 1).

Perfusion and function. All tests were assessed at baseline and at three-month follow-up. Myocardial perfusion was assessed by dual isotope SPECT imaging using thallium-201 for rest and technetium-99-sestamibi for adenosine-stress imaging. A semiquantitative (score 0 to 4) 17-segment model was used for analysis (7). Reversible ischemia was defined per segment as stress-rest score ≥ 1. Transthoracic echocardiography was used to assess global LV function and regional wall motion (score 1 to 4) using identical segmentation. Images were interpreted by core laboratories blind to injection location, study sequence, and patient clinical status. Change in perfusion was determined by grouping and averaging stress score of all segments with evidence of reversible ischemia at baseline lying within the entire area of distribution of each coronary artery, whether or not a given segment was injected. Exercise capacity was assessed using a modified Beth-Israel/Bruce treadmill protocol.

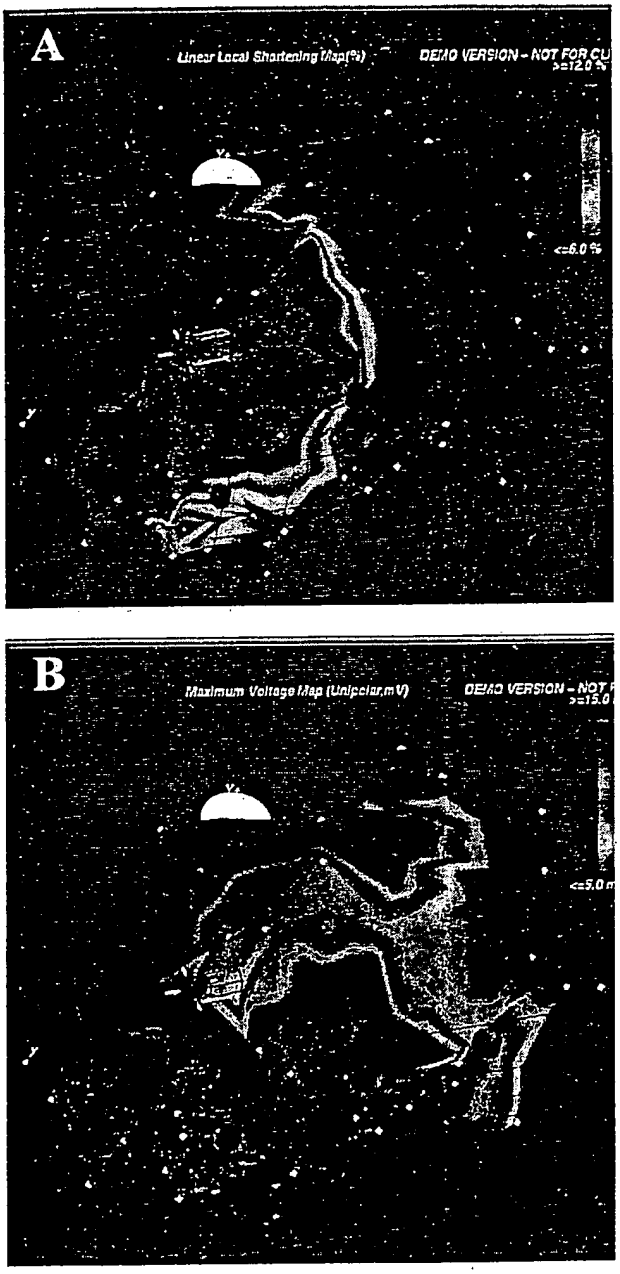

Figure 1. Left ventricular electromechanical maps (left oblique, bottom view projection) after 12 intramyocardial injections of autologous bone marrow. Injections were delivered into an ischemic right coronary artery territory. Local shortening (A) and unipolar voltage (B) amplitudes are color-coded and the exact location of each of the injection sites is tagged on the maps in real-time (black tags).

Patient follow-up. Echocardiography was performed within 2 h postprocedure. Serial blood samples for creatine kinase (CK)-MB were drawn and 12-lead electrocardiograms were acquired over 24 h postprocedure. Follow-up included CK-MB, complete blood count, physical examination, and event assessment. Bone marrow cultures for bacteria and fungi were followed for one month. Changes from baseline to three months in CCS angina class and

Table 1. Baseline Demographics and Clinical Data

Characteristics	Mean ± SD or n (%)
Mean age (yrs)	55 ± 7
Male gender	7 (70)
Angina class III	9 (90)
Angina class IV	1 (10)
Systemic hypertension	6 (60)
Diabetes mellitus	5 (50)
Insulin-dependent	1 (10)
Non–insulin-dependent	4 (40)
Hyperlipidemia	9 (90)
Smoking	8 (80)
Ejection fraction (%)	47 ± 10
Prior myocardial infarction	8 (80)
Prior bypass surgery	9 (90)
Prior PCI	10 (100)
Premature coronary artery disease	4 (40)
Nitrates	8 (80)
Beta-blockers	9 (90)
Calcium antagonists	6 (60)
Aspirin	10 (100)
Plavix	5 (50)
HMG-CoA reductase inhibitors	9 (90)

HMG-CoA = hydroxymethylglutaryl coenzyme A; PCI = percutaneous coronary intervention.

quality of life assessed by the Seattle Angina Questionnaire (8) were measured.

Statistical analysis. Data are presented as mean ± SD. Quantitative data were compared using paired, two-tailed Student *t* test. Qualitative (categorical) data, presented as frequencies, were compared using chi-square statistics. Repeated measure of variance was used to compare the concentration of VEGF and MCP-1 in the BM-conditioned medium collected at weeks 1, 2, 3, and 4. One-way analysis of variance was used to compare the effects of conditioned medium on HUVECs proliferation. A value of $p < 0.05$ was considered significant.

RESULTS

Patients. Baseline demographics and clinical data of the patients are summarized in Table 1.

BM. Cell viability was ≥95% in all patients. The filtered BM had no clots or bone spicules, had normal morphology, and stained negative for bacteria. The BM aspiration and processing time was approximated 2.5 h.

The injected BM contained 32.6 ± 27.5×10^6/ml nucleated cells with the following cell fraction: polymorphonuclear cells 74.6 ± 6.5%, lymphocytes 19.3 ± 8.1%, monocytes 3.5 ± 1.0%, and megakaryocytes 2.6 ± 2.3%. The CD34+ fraction was 2.6 ± 1.6%, of which 47.9 ± 15.1% co-expressed CD45. Among the double positive (CD45/CD34), 85 ± 14% co-expressed the stem cell factor receptor CD117 (c-kit).

Secretion of angiogenic growth factors by human BM cells. Over a four-week period VEGF and MCP-1 levels in the conditioned medium increased gradually from 0 to 6,472 ± 2,448 ng/ml ($p < 0.001$) and 2,094 ± 293 ng/ml ($p < 0.001$), respectively. The BM conditioned medium collected at four weeks increased, in a dose-related manner (10, 50, and 100 μl), proliferation of HUVECs by 25%, 50%, and 100% compared with controls ($p < 0.001$).

Procedural data. All patients were successfully injected with 12 injections. Left ventricular mapping and injection were associated with induction of ventricular premature beats, but no sustained ventricular or other arrhythmia occurred, and no significant changes were noted in blood pressure and heart rate.

Average mapping and BM injection procedure time was 30 ± 13 min and 28 ± 9 min, respectively. Overall, 45 segments were injected with an average of 4.5 ± 1.1 injections per segment with a mean distance of 12 ± 2 mm between injection points.

Clinical outcomes. There were no deaths, myocardial infarction, pericardial effusion, revascularization procedures, or stroke. None of the patients experienced endocarditis, myocarditis, or systemic infection, and all BM cultures were negative for bacteria and fungi. In-hospital peak CK-MB was within normal range in five patients and mildly elevated (1.1 to 1.9 times upper normal) in the remaining. Troponin I, available in eight patients, ranged between 0.16 and 0.62 ng/ml (infarction cutoff >0.8 ng/ml). All patients but one were discharged within 24 h of the procedure. In this patient a difficulty in advancing the mapping catheter was noted; the procedure was completed successfully via the other groin. Postprocedure magnetic resonance imaging revealed confined thoracic-abdominal dissection. The patient was followed conservatively and was discharged after three days. Three patients were readmitted to the hospital: one for atypical and two for typical chest pains not associated with electrocardiographic changes or myocardial enzyme elevation.

At three months, angina symptoms improved in eight patients and did not change in two patients, (CCS angina class 3.1 ± 0.3 vs. 2.0 ± 0.94, $p = 0.001$). Similar improvement was noted in angina stability score (15.6 ± 22.9 vs. 62.5 ± 32.7, $p = 0.002$), angina frequency (21.3 ± 27.0 vs. 32.5 ± 24.3 $p = 0.03$), and quality of life (26.0 ± 17.5 vs. 46.9 ± 23.1, $p = .0.01$).

Myocardial perfusion and function. At baseline, a total of 62 segments had reversible ischemia: 52 within territories subsequently injected (left anterior descending artery territory = 19, left circumflex artery = 23, and right coronary artery = 10) and 10 within noninjected (remote) territories. Stress score improved in segments within the injected but not within remote territories (Fig. 2), whereas rest score of these segments remained unchanged (0.25 ± 0.56 vs. 0.23 ± 0.47, $p = 0.85$). There was no change in EF (47 ± 10% vs. 52 ± 6%, $p = 0.24$). The majority of segments within the injected territories (n = 91, 80%) had normal baseline wall motion score, and none showed deterioration; among segments with abnormal baseline score, 2 improved and 21 showed no change. Exercise duration time, in the nine

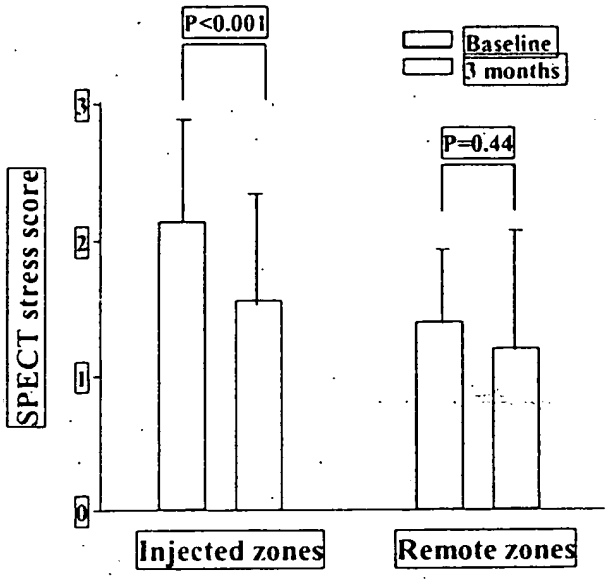

Figure 2. Semiquantitative stress scores of segments with baseline reversible ischemia within injected and remote coronary artery territories. SPECT = single-photon emission computed tomography.

patients undergoing this evaluation, increased from 391 ± 155 to 485 ± 198 (p = 0.11).

DISCUSSION

The current study is the first to assess the feasibility and potential safety of stand-alone transendocardial delivery of unfractionated ABM cells in patients with advanced coronary artery disease. Autologous bone marrow injection was associated with no serious adverse effects; in particular, there was no arrhythmia, evidence of infection, myocardial inflammation, or increased fibrosis. The study also suggests potential efficacy. However, it must be emphasized that no definitive safety- or efficacy-related conclusions can be drawn given the small number of patients and the study design.

We employed the ABM strategy because the BM contains diverse cells capable of secreting numerous cytokines and chemokines (3,4) as well as various progenitor and stem cells. The activity of these cells could potentially induce deleterious myocardial effects. We observed, however, no evidence of myocarditis and no adverse effect on regional myocardial perfusion or function. These results are in accord with our porcine study (4), and with a preliminary report of transepicardial injection of BM-derived mononuclear cells as an adjunct to bypass grafting surgery (9).

The magnitude of clinical improvement observed in the current study is in accord with previous trials of therapeutic angiogenesis (1,10). However, the variable outcome of angina symptoms among these patients, including spontaneous improvement, underscores the need for a large cohort-study to control for time-dependent natural variability and potentially important placebo effect.

The perfusion improvement we observed was in stress, but not in rest perfusion. This is in accord with a recent study employing transendocardial (6) delivery of VEGF, but not with a study employing intracoronary delivery of VEGF (11). The improvement we measured, however, may be fortuitous and due to the relatively small number of patients studied or to natural biologic variability in myocardial blood flow, rather than to treatment effect.

In conclusion, transendocardial administration of freshly aspirated ABM is potentially feasible. The results do not prove efficacy. We believe, however, that they do warrant larger controlled blinded studies, and should stimulate further investigational efforts to optimize this cell-based approach to enhancing collateral flow.

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Marrow-Derived Stromal Cells Express Genes Encoding a Broad Spectrum of Arteriogenic Cytokines and Promote In Vitro and In Vivo Arteriogenesis Through Paracrine Mechanisms

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Abstract—We recently demonstrated that marrow stromal cells (MSCs) augment collateral remodeling through release of several cytokines such as VEGF and bFGF rather than via cell incorporation into new or remodeling vessels. The present study was designed to characterize the full spectrum of cytokine genes expressed by MSCs and to further examine the role of paracrine mechanisms that underpin their therapeutic potential. Normal human MSCs were cultured under normoxic or hypoxic conditions for 72 hours. The gene expression profile of the cells was determined using Affymetrix GeneChips representing 12 000 genes. A wide array of arteriogenic cytokine genes were expressed at baseline, and several were induced >1.5-fold by hypoxic stress. The gene array data were confirmed using ELISA assays and immunoblotting of the MSC conditioned media (MSC^{CM}). MSC^{CM} promoted in vitro proliferation and migration of endothelial cells in a dose-dependent manner; anti-VEGF and anti-FGF antibodies only partially attenuated these effects. Similarly, MSC^{CM} promoted smooth muscle cell proliferation and migration in a dose-dependent manner. Using a murine hindlimb ischemia model, murine MSC^{CM} enhanced collateral flow recovery and remodeling, improved limb function, reduced the incidence of autoamputation, and attenuated muscle atrophy compared with control media. These data indicate that paracrine signaling is an important mediator of bone marrow cell therapy in tissue ischemia, and that cell incorporation into vessels is not a prerequisite for their effects. **(*Circ Res.* 2004;94:678-685.)**

Key Words: marrow stromal cells ■ arteriogenesis ■ bone marrow cells ■ cytokines

An important compensatory response to atherosclerotic obstructive arterial disease is collateral development, a complex process requiring that multiple genes coordinately express their products in an appropriate time-dependent manner.[1,2] However, the natural capacity of collaterals to remodel and enlarge to compensate for the reduced flow that occurs after occlusion of a major artery is rarely sufficient to restore maximal flow capacity to levels required under various stress-conditions.

Although several protein and gene-based strategies have succeeded in enhancing collateral development in animal models of ischemia, clinical studies thus far have been disappointing.[3–5] Given that the natural response to tissue ischemia is such a complex process, the delivery of a single growth factor may be too simple an approach. Thus, a great deal of interest has arisen in the potential of cell-based strategies in augmenting collateral responses, and several groups have demonstrated incorporation of various bone marrow–derived cells into new or remodeling vessels.[6,7]

However, the actual magnitude of incorporation of bone marrow–derived cells into vascular structures varies substantially between studies. Although some studies report over 50% of capillaries containing transplanted cells, other studies have reported only occasional positive vessels despite impressive improvement in perfusion.[8–10] Taken together, these data suggest that other mechanisms apart from cell incorporation may contribute to collateral remodeling observed after bone marrow–derived cell therapy in various models of ischemia. Furthermore, we recently demonstrated that marrow stromal cells (MSCs) augment collateral remodeling through release of several cytokines such as VEGF and bFGF rather than via cell incorporation into new or remodeling vessels.[11] Therefore, the purpose of the present study is to characterize the full spectrum of cytokine genes expressed by MSCs and to further examine the role of paracrine mechanisms that underpin the biological effects of MSC therapy for tissue ischemia.

Materials and Methods

Human Cell Lines

Normal human MSCs (derived from a single 19-year-old healthy donor) were purchased from Clonetics (Walkersville, Md) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (DM-10). These cells were previously demonstrated to

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be negative for CD34 and CD45 surface markers, and positive for CD44, CD105, and CD166. Passages 3 to 5 were used for in vitro experiments. Human umbilical vein endothelial cells (ECs) were purchased from American Type Culture Collection (ATCC, Manassas, Va) and cultured in endothelial growth media-2 (EGM-2; Clonetics). Human aortic smooth muscle cells (SMCs) were purchased from ATCC and cultured in Medium-199 supplemented with 10% fetal bovine medium and 1% penicillin-streptomycin (M-10). Passages 3 to 8 were used for in vitro experiments. Cells were cultured in 20% O_2 and 5% CO_2 during normoxia experiments, and in 1% O_2 and 5% CO_2 using a hypoxia chamber for hypoxia experiments.

RNA Preparation

Total RNA was extracted from normoxia and hypoxia exposed (72 hours) human MSCs (2 plates per analysis) using TRIzol Reagent (Invitrogen) according to the manufacture's instructions. RNA was cleaned using a RNeasy mini kit (Qiagen) and stored at −80°C.

Microarray Analysis

Double-stranded cDNA was synthesized from 8 μg of total RNA. For the first cDNA strand synthesis, oligo(dT) primers were annealed to the RNA and extension by reverse transcriptase was performed in the presence of deoxyoligonucleotides. The second strand was synthesized using DNA polymerase I and purified using a phase lock gels-phenol/chloroform extraction, followed by ethanol precipitation. In vitro transcription, using double-stranded cDNA as a template in the presence of biotin-labeled ribonucleotides, was performed by using an Enzo in vitro transcription kit (Enzo Diagnostics). Biotin-labeled cRNA was purified, fragmented, and hybridized to Affymetrix Human Genome U133A GeneChips (Affymetrix, Santa Clara, Calif). Hybridization, washing, antibody amplification, staining, and scanning of probe arrays were performed according to the Affymetrix Technical manual. Scanned raw data were processed with Affymetrix GeneChip v 5.1 software. A hypoxia fold induction of >1.5 was considered significant.

Human Conditioned Media Collection, Preparation, and Analysis

For ELISA, human MSC-conditioned media ($hMSC^{CM}$) was collected after 24 hours of culture, centrifuged at 2000 rpm for 10 minutes, and passed through a 0.3 μm filter. The concentration of MSC^{CM} cytokines was measured using sandwich ELISA kits (VEGF, bFGF, MCP-1, and PlGF; R&D systems). After media collection, cells were lysed and total protein counted with the MicroBCA protein assay (Pierce). ELISA values were corrected for total cell protein. EC-conditioned media and DM-10 were also assayed. For cell proliferation and migration assays, $hMSC^{CM}$ was collected for 72 hours and filtered as above. Dilutions were prepared with DM-10 as appropriate.

For immunoblotting, $hMSC^{CM}$ was collected and prepared as above. Forty micrograms of protein were separated using SDS-PAGE gels (10%) and blotted onto nitrocellulose (Invitrogen). After blocking, blots were incubated with primary antibody to PDGF-β (1:1000, Santa Cruz), angiopoetin-1 (1:1500, Santa Cruz), metalloproteinase-9 (1:500, Santa Cruz), or plasminogen activator (1:1000, Santa Cruz). Membranes were developed with an enhanced chemiluminescence kit (Pierce).

Cell Proliferation Assay

ECs or SMCs (1×10^4/well) were plated in 24-well plates in DMEM with 0.1% fetal calf serum for 24 hours to arrest mitosis. For EC proliferation, the media was replaced with varying dilutions of $hMSC^{CM}$, EGM-2 (positive control), recombinant VEGF 4 ng/mL (positive control, R&D Systems), DM-10 (normal control), or boiled $hMSC^{CM}$ (negative control). Further DM-10 samples were supplemented with recombinant VEGF to concentrations coinciding with the concentration of VEGF present in the conditioned medium. To examine the role of cytokines in isolation, 10 μg/mL anti-VEGF antibody (Sigma), 5 μg/mL anti-FGF antibody (Sigma), or 5 μg/mL anti-PDGF antibody (Sigma) was added to $hMSC^{CM}$ dilutions in additional wells. Further DM-10 samples were also supplemented with recombinant VEGF to concentrations found in the relevant dilutions of $hMSC^{CM}$. For SMC proliferation, the media was replaced with varying dilutions of MSC^{CM}, PDGF (10 ng/mL, positive control, Clonetics), or DM-10 (normal control). Cultures were continued for 72 hours, after which the cells were recovered and counted using a Coulter counter. Data are reported as the mean percentage change in proliferation when compared with control media (DM-10).

Cell Migration Assay

EC and SMC migration assays were performed using Transwell culture chambers (Costar, Corning). Cells were suspended in DMEM supplemented with 2% serum and placed in the top chamber (4×10^5/well). For EC migration, DM-10 (normal control), $hMSC^{CM}$, boiled $hMSC^{CM}$, and VEGF (4 ng/mL, positive control) were added to the lower chamber. For SMC migration, DM-10, $hMSC^{CM}$, boiled $hMSC^{CM}$, and PDGF (10 ng/mL, positive control) were added to the lower chamber. Cells were incubated overnight, and the top layer of the membrane scraped gently to remove any cells. Cells on the lower surface of the membrane were stained using Hema-3 staining kit (Biochemical Sciences). Six random fields per membrane were counted. Data are reported as the mean percentage or fold change in proliferation when compared with control media (DM-10).

Murine MSC Preparation and Culture

Murine bone marrow was harvested by flushing the tibiae and femurs of Balb/C mice (two mice per culture; Jackson Laboratories, Bar Harbor, Maine) with DM-10. The pooled marrow was dispersed, plated in DM-10, and cultured for 72 hours. Nonadherent cells were washed off and adherent cells expanded until confluent (≈7 to 10 days). FACS analysis of up to passage 6 demonstrated persistence of lymphohematopoietic cells ($CD34^+$ or $CD45^+$ or both). Therefore, MSCs were purified from the heterogeneous cultured cells. The $CD34^-/CD45^-$ fraction was isolated by labeling with FITC-conjugated anti-CD34 antibody (Pharmingen) followed by simultaneous incubation with a cocktail of anti-FITC and anti-CD45 magnetic beads (Miltenyi Biotech). Cells were passed through a magnetic column, the double-negative fraction collected, and replated. Repeat FACS analysis was performed and demonstrated that cells did not express CD31, CD34, CD45, and CD117, and expressed high levels of CD44, CD90, and CD105 (data not shown) typical of marrow-derived stromal cells, and in keeping with previous published data.[12] For in vivo experiments, murine MSC^{CM} ($mMSC^{CM}$) was collected after 72 hours and then concentrated 2-fold using Microcon YM-10 centrifugal filters (Amicon). As a control, DM-10 was filtered and concentrated in a likewise fashion.

Animal Surgery and Murine MSC^{CM} injection

All animal interventions were approved by the Animal Care and Use Committee of the MedStar Research Institute. Under narcosis, 12-week-old Balb/C mice (n=5 per group) were subjected to operative intervention to create unilateral hindlimb ischemia. The right femoral artery was exposed in the midthigh, dissected from the femoral vein and nerve, and then ligated just proximal to the popliteal bifurcation. In preliminary studies, MSC^{CM} injection immediately after femoral ligation failed to improve flow recovery. Therefore, in the present study, $mMSC^{CM}$ injection was delayed by 24 hours to allow the mice to partially recover from the surgical insult. A total of 50 μL of $mMSC^{CM}$ or DM-10 was injected into the adductor muscle at four sites adjacent to and within 1 mm of the ligation site. The injections were repeated at 48 hours and 72 hours.

Perfusion Imaging

Laser doppler perfusion imaging (LDPI) (Moor Instruments) was utilized to record serial blood flow measurements. For consistent measurements, imaging was performed after limb hair was removed, and after mice had been placed on a heating plate at 37°C to minimize temperature variation. Calculated perfusion is expressed as a ratio of the ischemic to normal limb.[13] Previous data have

Marrow-Derived Stromal Cells Proangiogenic/Proarteriogenic Gene Expression

Cytokine	Angiogenic/Arteriogenic Function	Fold Induction With Hypoxia
Angiopoetin-1	EC migration, vessel stabilization	...
Fibroblast growth factor-2	EC and SMC proliferation and migration	1.62
Fibroblast growth factor-7	EC proliferation and stabilization	1.82
Hepatoma growth factor	SMC proliferation	...
Interleukin-1	VEGF induction	1.91
Interleukin-6	VEGF induction	2.26
Metalloproteinase-1	Loosens matrix, tubule formation	...
Metalloproteinase-2	Loosens matrix, tubule formation	...
Metalloproteinase-9	Loosens matrix	...
MCP-1	Monocyte migration	...
M-CSF	Monocyte proliferation/migration	...
Placental growth factor	EC proliferation	2.93
Plasminogen activator	Degrading matrix molecules	...
Platelet-derived growth factor	SMC proliferation and migration	...
Stem cell–derived factor	Progenitor cell homing	...
Transforming growth factor-β	Vessel maturation, EC proliferation	2.11
Tumor necrosis factor-α	Degrade matrix molecules, EC proliferation	1.69
VEGF-A	EC proliferation, migration, tube formation	2.47
VEGF-B	EC proliferation, migration, tube formation	...

MCP-1 indicates monocyte chemoattractant protein-1; M-CSF, macrophage-specific colony-stimulating factor; VEGF, vascular endothelial growth factor; EC, endothelial cell; and SMC, smooth muscle cell.

suggested a close linear relationship between recovery of perfusion as assessed by LDPI, and positive remodeling of adductor collateral vessels.[14]

In Vivo Assessment of Limb Function

Functional assessment of the ischemic limb was performed using a modification of a clinical standard score.[15] A semiquantitative assessment of ambulatory impairment of the ischemic limb was performed serially (0=flexing the toes to resist gentle traction on the tail, 1=plantar flexion, 2=no dragging but no plantar flexion, 3=dragging of foot). A blinded observer assigned all scores.

Histological Assessment of Collateral Morphology

After completing blood flow assessment, sections of adductor muscles were stained with van Gieson's solution. Only arteries, identified by the presence of a continuous internal elastic laminae and muscle spindles, and with a mathematically derived area >300 μm^2, were counted. Total cross sectional area was calculated using Image-Pro software, with the smallest internal luminal distance measured as the radius.

Data and Statistical Analysis

Expression analysis data were verified by performing experiments in duplicate. All ELISA, immunoblotting, and cell studies were performed at least in triplicate. All results are presented as mean±SEM. Statistical significance was evaluated using an unpaired student *t* test, or ANOVA where indicated. A value of $P<0.05$ was considered significant.

Results

Gene Array of Human MSCs

MSCs expressed genes for a wide array of arteriogenic cytokines (Table). The expression of FGF-2, FGF-7, interleukin-1 and interleukin-6, placental growth factor, TGF-β, TNF-α, and VEGF-A were all augmented by exposure to hypoxic stress.

Cytokine Release From MSCs

To complement gene expression patterns, we analyzed the cytokine content of $hMSC^{CM}$ using ELISA (Figure 1). Baseline and hypoxic augmentation of VEGF-A secretion (375 pg/mg protein in normoxia versus 698 pg/mg in hypoxia; $P<0.01$) was confirmed with a similar pattern also seen for FGF-2 (2320 pg/mg versus 3970 pg/mg; $P<0.05$), and interleukin-6 (3885 pg/mg versus 7665 pg/mg; $P<0.01$), reflecting similar changes in gene expression. Although placental growth factor gene expression was augmented 3-fold by hypoxic stress, secretion of the cytokine was not significantly altered (119 pg/mg versus 164 pg; P=NS). Monocyte chemoattractant protein-1 gene expression was unchanged after exposure to hypoxic stress and a similar pattern of secretion was also seen (150 pg/mg versus 70 pg/mg; P=NS). Immunoblotting of the MSC^{CM} for angiopoetin-1, PDGF, metalloproteinase-9, and plasminogen activator also demonstrated similar cytokine release profiles to the expression profiles (Figure 2). ECs released minimal cytokines under baseline conditions and in response to hypoxia.

$hMSC^{CM}$ Effect on Endothelial Cell Proliferation and Migration

To examine whether $hMSC^{CM}$ exhibited biological effects relevant to collateral remodeling, a series of EC proliferation assays were performed. $hMSC^{CM}$ significantly enhanced EC

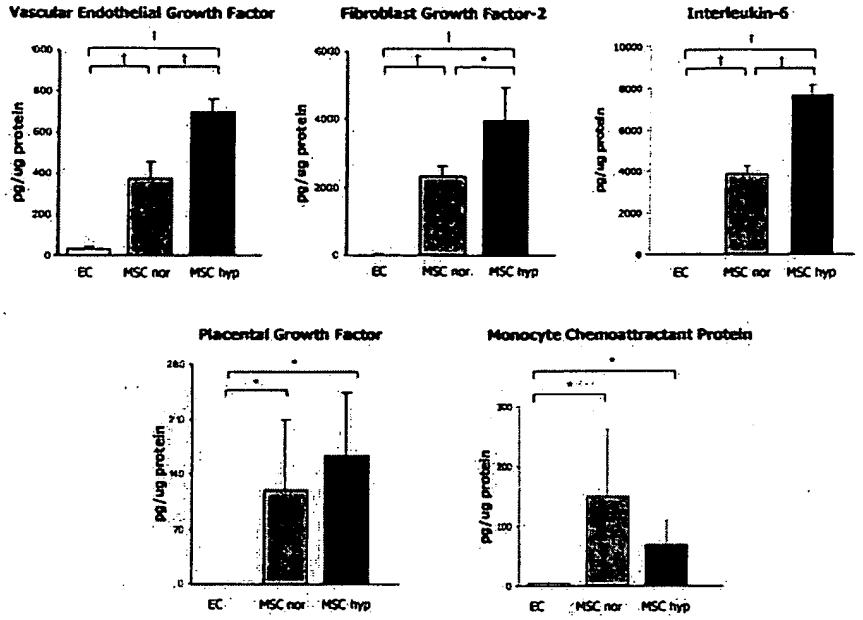

Figure 1. Cytokines released by MSCs in culture. In vitro release of VEGF, bFGF, IL-6, PIGF, and MCP-1 from marrow stromal cells as assessed by ELISA analysis of hMSC^{CM}. Data displayed from control ECs, hMSCs in normoxia, and hMSCs under hypoxic stress. *$P<0.05$, †$P<0.01$.

proliferation over control and was comparable to growth seen with EGM-2 (Figure 3A). The proliferative effect of hMSC^{CM} was completely abolished by boiling, suggesting these effects were due to a specific receptor/ligand interaction. As expected, the mitogenic effect of recombinant VEGF was inhibited by the addition of VEGF-blocking antibody (Figure 3A). In contrast to this, addition of the same antibody to hMSC^{CM} only partially attenuated EC proliferation (5.5-fold without antibody versus 3.6-fold with antibody; $P<0.001$; Figure 3B). Similarly, addition of an FGF-2 blocking antibody only partly attenuated proliferation (5.5-fold versus 4.6-fold, respectively; $P<0.05$, $P<0.001$ versus control media). The addition of both blocking antibodies significantly reduced the mitogenic effects, although the effect was still significant over control ($P<0.001$). There was no significant change in EC proliferation in response to hMSC^{CM} following the addition of anti-PDGF antibody. Additionally, reconstitution of DM-10 with recombinant VEGF to concentrations seen in the hMSC^{CM} dilutions failed to stimulate EC proliferation to the same extent as hMSC^{CM} (Figure 3C). The data strongly suggest that the mitogenic effects of hMSC^{CM} are due to multiple cytokines. Finally, a hMSC^{CM} dose-response relationship was also demonstrated (Figure 3C).

To further examine the biological effects of hMSC^{CM}, its effect on EC migration was studied. hMSC^{CM} induced a 5.5-fold increase in EC migration compared with control, although this did not achieve the fold increase observed with recombinant VEGF (Figures 4A and 4B). As with EC proliferation, boiling eliminated the chemoattractant properties of hMSC^{CM}.

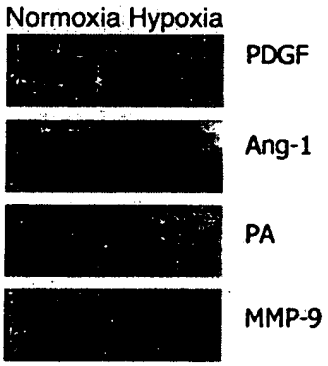

Figure 2. Immunoblotting of hMSC^{CM} levels of angiopoetin-1 (ang-1), platelet-derived growth factor (PDGF), metalloproteinase-9 (MMP-9), and plasminogen activator (PA).

hMSC^{CM} Effect on Smooth Muscle Cell Proliferation and Migration

hMSC^{CM} stimulated proliferation of SMCs in a dose-responsive manner, although this did not reach the effect seen with recombinant PDGF-β. As in ECs cultures, the SMC proliferative effect of hMSC^{CM} was abolished by boiling (Figure 5A). hMSC^{CM} also exerted a chemoattractant effect on SMCs, although the effect was only weak in comparison to PDGF-β and was inhibited by boiling (Figure 5B).

Hindlimb Blood Flow, Limb Recovery, and Collateral Morphology After mMSC^{CM} Injection

Having established that MSCs secrete many arteriogenic cytokines, and that the hMSC^{CM} exerts in vitro biological effects relevant to collateral remodeling, we proceeded to examine whether, as part of their therapeutic benefit, MSCs were able to contribute to collateral remodeling through paracrine mechanisms. To do this, we injected mMSC^{CM} directly into the adductor muscle (area of collateral remodeling) in a mouse model of hindlimb ischemia. In mice receiving control media, flow returned to ≈50% of the nonischemic limb by day 28. In contrast, in those mice receiving mMSC^{CM} there was a significant improvement in flow (Figure 6A) by day 3, which was maintained for the duration of the study ($P<0.05$ by ANOVA). Representative flow images are displayed in Figure 6B. In mice receiving MSC^{CM}, total arterial cross sectional area was significantly

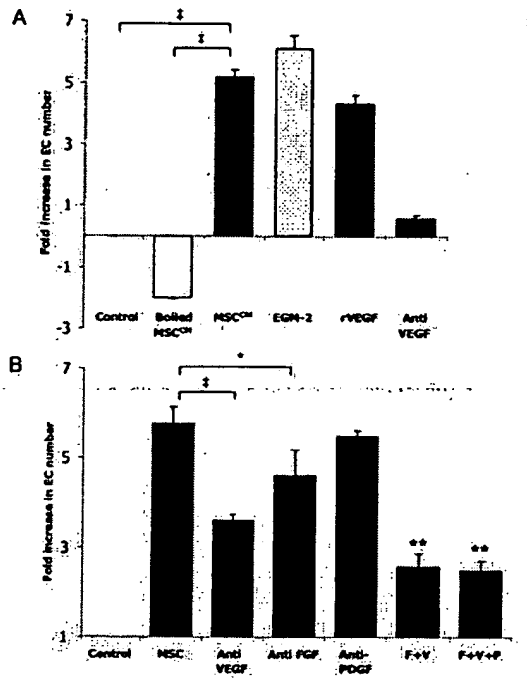

Figure 3. In vitro effects of hMSC^{CM} on EC proliferation and migration. A, Percent increase in the number of ECs over control media. EC proliferation with hMSC^{CM} approaches that seen with a commercial endothelial cell growth medium (EGM-2); this effect is abolished by boiling. Recombinant VEGF (rVEGF, 10 ng/mL) is used as a positive control and can be inhibited by the addition of blocking antibody. ‡$P<0.001$. B, Effects of blocking antibodies to VEGF (anti-VEGF, 10 μg/mL), FGF-2 (anti-FGF, 5 μg/mL), and PDGF (anti-PDGF, 5 μg/mL) on the EC mitogenic effects of hMSC^{CM}. Blocking VEGF or FGF-2 partially attenuated the response, although even the addition of both failed to totally abrogate the hMSC^{CM} effects (F+V, **$P<0.001$ vs control media). C, Graph demonstrating a dose-response curve between hMSC^{CM} dilutions and EC proliferation (filled squares). Reconstitution of control media with recombinant VEGF to the same concentrations as in the hMSC^{CM} dilutions failed to stimulate EC proliferation to the same degree (open squares). As before, addition of the same blocking VEGF antibody to the MSC^{CM} only partially attenuated proliferation (open circles).

increased in those mice compared with control (8380 μm^2 versus 4303 μm^2; $P<0.05$; Figure 6C).

The improved flow recovery was associated with improved hindlimb appearance and function. Mice receiving control media experienced severe ischemic damage resulting in a 60% incidence of autoamputation by day 28. However, mice receiving mMSC^{CM} displayed less ischemic damage with a 20% autoamputation rate. Similarly, in mice receiving mMSC^{CM}, limb function was significantly better than those mice receiving control media (ambulatory score 2.25 versus 1.25, respectively; $P<0.05$; Figure 6D).

Improved flow recovery also attenuated the calf muscle atrophy observed after femoral artery ligation. In control mice, muscle loss was significantly greater than in those mice receiving mMSC^{CM} (69% versus 41%, respectively; $P<0.05$; Figure 6E).

Discussion

Cells of the marrow stroma maintain hematopoietic stem cells and their progeny through a variety of molecular mechanisms including direct cell-to-cell interactions and, importantly, through local release of supportive cytokines.[16,17] Isolated reports have previously demonstrated release of VEGF and bFGF, but the present study is the first to our knowledge to definitively characterize the full spectrum of arteriogenic cytokines released by marrow-derived stromal cells. In addition, previous studies have documented marrow stromal cell secretion of hepatocyte growth factor,[18] insulin-like growth factor,[19] and MCP-2/MCP-3,[20] although mRNA for these cytokines was not found in the present study. Importantly, hypoxia also led to increases in the mRNA expression and secretion of several important cytokines such as VEGF and FGF-2 without adversely affecting release of any other cytokines. This is of relevance as the milieu into which cells are injected, such as ischemia versus nonischemia, is likely to have a major influence on their subsequent behavior.

The ability of bone marrow cells to secrete multiple arteriogenic cytokines has led to several studies demonstrating these cells enhance collateral flow, and the responsible mechanism has often been ascribed to these cells incorporating into the developing collaterals. However, the actual magnitude of incorporation of bone marrow–derived cells into vascular structures varies substantially among studies. Although some studies report over 50% of capillaries con-

Figure 4. A, EC chemoattractant properties of hMSC^{CM}. There is a significant increase in EC migration seen with hMSC^{CM}, although it is less than that seen with recombinant VEGF (rVEGF, 10 ng/mL) control. †$P<0.01$, ‡$P<0.001$. B, Representative Transwell membranes stained with Hema-3, illustrating significant increase in EC migration compared with control.

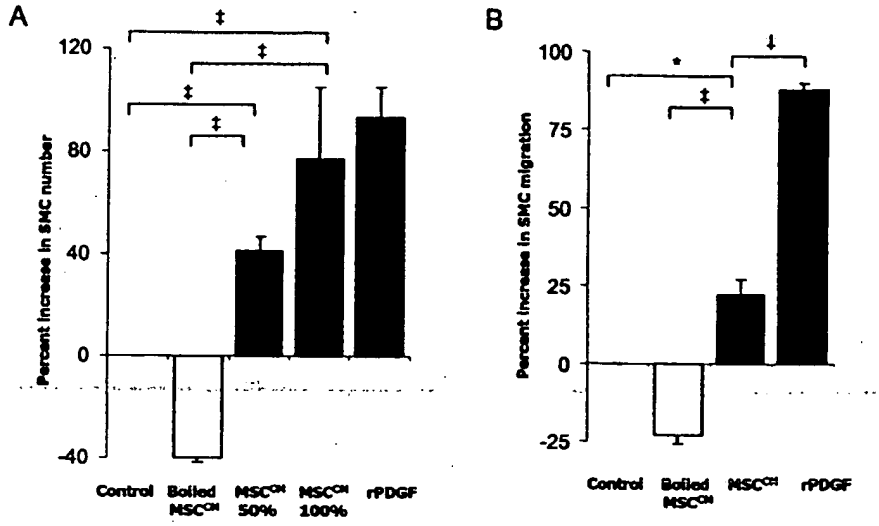

Figure 5. In vitro effects of hMSC^{CM} on SMC proliferation and migration. A, Percent increase in the number of SMCs over control media. Dose response is apparent as indicated by 50% and 100% dilutions of hMSC^{CM}, and this effect is abolished by boiling. Proliferation with recombinant PDGF (rPDGF, 10 ng/mL) as a positive control. ‡$P<0.001$. B, SMC chemoattractant properties of hMSC^{CM}. Mild increase in SMC migration seen with hMSC^{CM}, which is abolished by boiling. Chemoattractant effects seen do not reach that observed with recombinant PDGF control (rPDGF, 10 ng/mL). *$P<0.05$, ‡$P<0.001$.

taining transplanted cells, other studies have reported only occasional positive vessels despite noting impressive improvements in perfusion.[8–10] Taken together, these data suggest that other mechanisms apart from cell incorporation contribute to collateral remodeling observed after bone marrow–derived cell therapy in various models of ischemia.

The present study demonstrates that numerous arteriogenic cytokines are released by MSCs and, importantly, that injection of cells themselves is not required for therapeutic benefit, but that the release of such cytokines is sufficient to mediate arteriogenesis and enhance collateral flow. However, it is likely that complimentary mechanisms may contribute to the beneficial effects on blood vessel formation seen after cell therapy. Marrow stromal cells—also termed mesenchymal stem cells—have been demonstrated to differentiate into smooth muscle and endothelial cell lineages,[21–24] and thus may contribute cells directly to new or remodeling vessels. Nonetheless, the importance of the mechanism is still controversial.

Cytokines have not only individual effects, but one cytokine may potentiate (or inhibit) the effect of another. A synergistic relationship between VEGF and bFGF was reported in a rabbit ischemic hindlimb model, and placental growth factor appears to potentiate the effects of VEGF, both in in vitro and in vivo models.[25,26] Other studies have demonstrated synergism between PDGF and FGF-2 as well as between angiopoietin-1 and VEGF.[27,28] The present study also demonstrates this synergism. Blocking the effects of VEGF and bFGF in MSC^{CM} only partly attenuates the mitogenic effects of the MSC^{CM} on endothelial cells. Reconstitution of control media with recombinant VEGF to similar levels as that found in the MSC^{CM} stimulates endothelial cell proliferation, but not nearly to the extent as achieved with whole MSC^{CM}. Taken together, these data imply that multiple cytokines secreted by MSCs have additive or synergistic effects on cell proliferation, and as such MSC therapy may be more effective than single protein approaches in augmenting

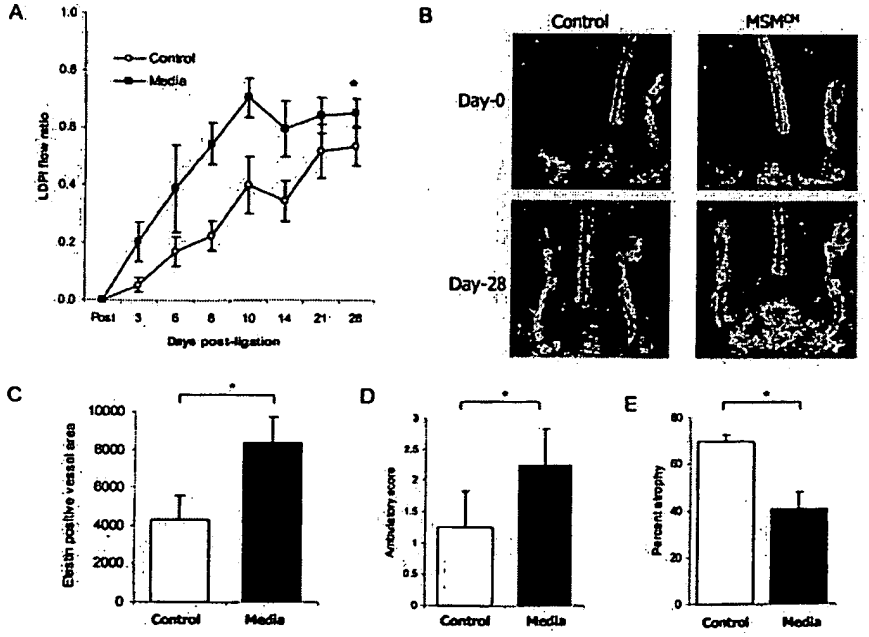

Figure 6. In vivo effects of mMSC^{CM}. A, Perfusion analysis after mMSC^{CM} treatment. LDPI expressed as a percentage of the normal limb. Flow recovery in mMSC^{CM}-treated animals was significantly better than that seen in animals treated with control media (*$P<0.05$ by ANOVA). B, Representative LDPI images of flow recovery in a mouse receiving mMSC^{CM} versus a mouse receiving control media. Red is highest velocity, green intermediate, and blue, lowest velocity. Postligation and day-28 images are displayed. C, Total cross-sectional area of elastin-positive vessels in the adductor muscle of mice treated with mMSC^{CM} versus control media (*$P<0.05$). D, MSC^{CM} injection led to a significant improvement of ambulatory function over control (*$P<0.05$). E, mMSC^{CM} injection significantly reduced calf atrophy versus control media (*$P<0.05$).

tissue perfusion. It is also interesting to speculate that MSC^{CM} could be used therapeutically rather than MSCs themselves, thus avoiding many practical issues regarding cell therapy.

Previous work examining the role of MSCs in angiogenesis demonstrated, using a Matrigel implantation model, that MSCs could augment capillary in-growth through paracrine mechanisms.[29] In that study, MSCs out to passage 14 were used, and their effects could be completely inhibited by addition of neutralizing anti-VEGF antibodies. These observations contrast with the present results. However, we have observed a gradual decrease over time in the release of PlGF and bFGF in MSC cultures (data not shown), whereas VEGF and MCP-1 levels remain relatively constant up to 4-weeks. Thus changes in the cytokine release profile over time may explain differences between this and previous studies. In the clinical setting, therefore, the timing of cell harvest may have important consequences for cell therapy in patients.

Given the importance of paracrine signaling in MSC/hematopoietic cell interactions, it is perhaps not surprising that MSC can augment collateral remodeling through paracrine mechanisms. However, previous studies have suggested this phenomenon may not be restricted to MSCs and that other bone marrow–derived cells may also influence blood flow recovery through release of arteriogenic cytokines. For example, bone marrow mononuclear cells contain mRNA for VEGF, bFGF, and angiopoietin-1, and after injection of BM mononuclear cells, local increases in VEGF protein have been observed.[30–32] Interestingly, injection of human-derived angioblasts into infarcted rat myocardium appeared to stimulate local host endothelial cells to proliferate, suggesting that these angioblasts may be a source of proangiogenic factors.[33] Endothelial progenitor cells in vitro also release several relevant cytokines, including VEGF and GM-CSF.[34] Thus, previous data in combination with the present study imply that bone marrow–derived progenitor cells can improve tissue ischemia in part through paracrine mechanisms. However, the exact degree to which this occurs is likely to vary from cell-to-cell and from milieu-to-milieu.

In summary, our data demonstrate that marrow-derived stromal cells secrete a broad spectrum of cytokines, which in vitro stimulate endothelial and smooth muscle cells to proliferate and migrate. These effects are dose-dependent and appear to be mediated by several cytokines. Furthermore, local injection of marrow stromal cell–derived conditioned media alone enhances collateral perfusion and remodeling in a murine model of hindlimb ischemia, reducing tissue atrophy and limb damage, and improving limb function, suggesting that paracrine signaling is an important mediator of bone marrow cell therapy in tissue ischemia.

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Local Delivery of Marrow-Derived Stromal Cells Augments Collateral Perfusion Through Paracrine Mechanisms

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Background—Bone marrow cell therapy is reported to contribute to collateral formation through cell incorporation into new or remodeling vessels. However, the possible role of a paracrine contribution to this effect is less well characterized.

Methods and Results—Murine marrow-derived stromal cells (MSCs) were purified by magnetic bead separation of cultured bone marrow. The release of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PlGF), and monocyte chemoattractant protein-1 (MCP-1) was demonstrated by analysis of MSC conditioned media (MSC-CM). MSC-CM enhanced proliferation of endothelial cells and smooth muscle cells in a dose-dependent manner; anti-VEGF and anti-FGF antibodies only partly attenuated these effects. Balb/C mice (n=10) underwent distal femoral artery ligation, followed by adductor muscle injection of 1×10^6 MSCs 24 hours later. Compared with controls injected with media (n=10) or mature endothelial cells (n=8), distal limb perfusion improved, and mid-thigh conductance vessels increased in number and total cross-sectional area. MSC injection improved limb function and appearance, reduced the incidence of auto-amputation, and attenuated muscle atrophy and fibrosis. After injection, labeled MSCs were seen dispersed between muscle fibers but were not seen incorporated into mature collaterals. Injection of MSCs increased adductor muscle levels of bFGF and VEGF protein compared with controls. Finally, colocalization of VEGF and transplanted MSCs within adductor tissue was demonstrated.

Conclusions—MSCs secrete a wide array of arteriogenic cytokines. MSCs can contribute to collateral remodeling through paracrine mechanisms. (*Circulation.* 2004;109:1543-1549.)

Key Words: cells, bone marrow ■ cells, stromal ■ angiogenesis

Several bone marrow subpopulations, such as endothelial progenitor cells and marrow stromal cell fraction (marrow-derived stromal cells [MSCs]), may be able to differentiate into 1 or more of the cellular components of the vascular bed.[1–3] Thus, therapeutic delivery of bone marrow donates cells with potential to incorporate into new or remodeling blood vessels. However, the magnitude of incorporation of bone marrow–derived cells into vascular structures varies between studies. Although >50% of capillaries containing transplanted cells have been reported, only a single transplanted cell in the circumference of the vessel is required for it to be counted as a positive vessel. Furthermore, other studies have reported small numbers of positive vessels, despite impressive improvements in perfusion.[4–6] Taken together, these data suggest that other mechanisms may contribute to the improved collateral perfusion observed after stem cell therapy in various models of ischemia.

MSCs play an important supportive role in the marrow microenvironment, mediated partly through cell-to-cell contact but importantly also via paracrine mechanisms involving release of cytokines that exert effects on surrounding cells. Therefore, the present study sought to examine the hypothesis that local delivery of MSCs augments collateral perfusion in a murine hindlimb ischemia model and that these effects are mediated by paracrine mechanisms rather than by cell incorporation.

Methods

MSC Harvest, Culture, and Isolation

Bone marrow was harvested by flushing the tibiae and femurs of Balb/C mice with DMEM supplemented with 10% fetal bovine medium (DM-10). The pooled marrow was plated in DM-10 supplemented with 1% penicillin-streptomycin and cultured for 72 hours. Nonadherent cells were washed off, and adherent cells expanded until confluent (~7 to 10 days).

MSCs were purified from the heterogeneous cultured cells. The CD34−/CD45− fraction was isolated by labeling with fluorescein isothiocyanate–labeled anti-CD34 antibody (Pharmingen), simultaneous incubation with anti–fluorescein isothiocyanate and anti-CD45 magnetic beads (Miltenyi Biotech), and passage through a magnetic column. Subsequently, the bead-negative and bead-positive populations were cultured separately. The bead-negative

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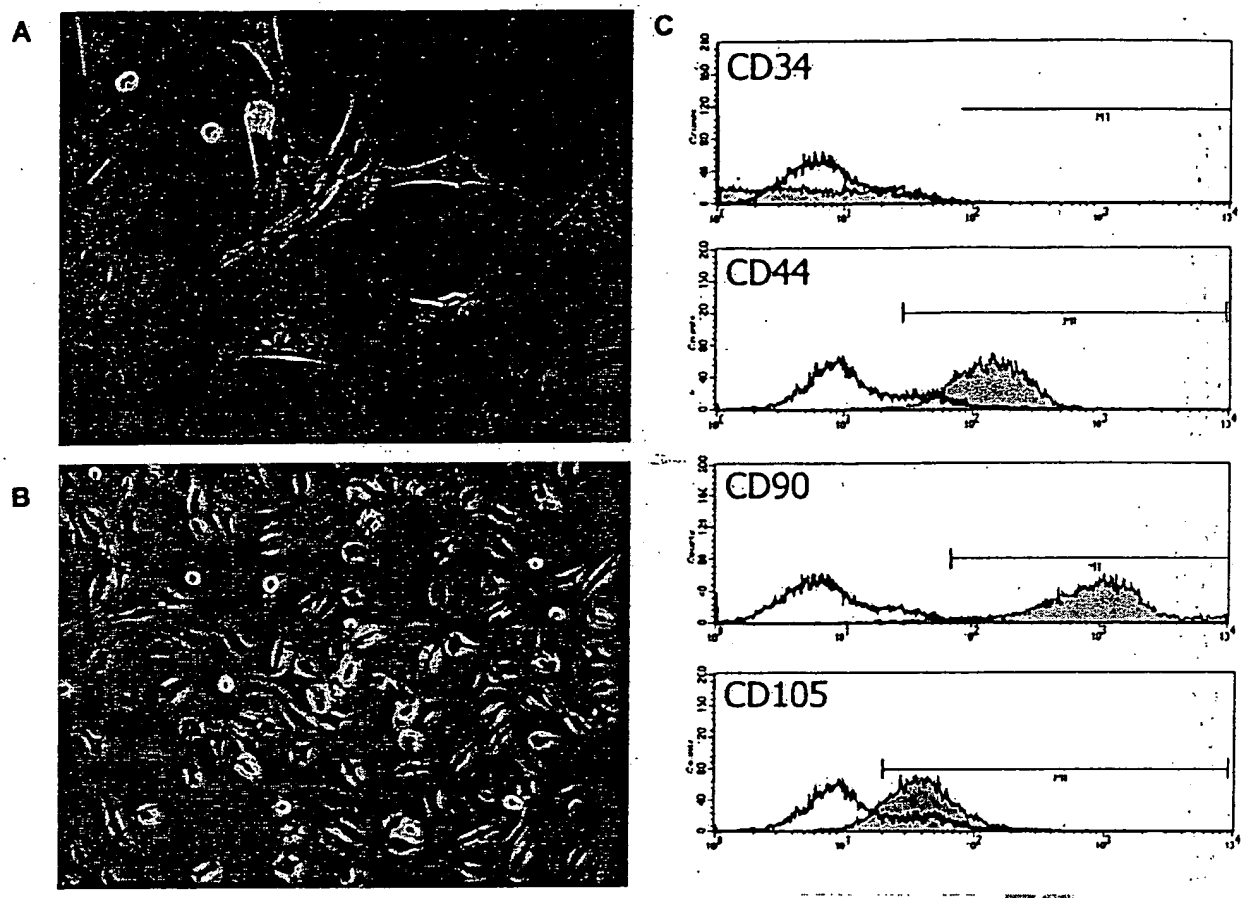

Figure 1. A, CD34−/CD45− cells show fibroblastic morphology typical of MSCs. B, CD34+/CD45+ cells show spherical morphology consistent with lymphohematopoietic cells. C, FACS analysis of murine MSCs. Cells were uniformly negative for CD34 and positive for CD44 (95±0.6%), CD90 (99.1±0.1%), and CD105 (89±2.1%), markers associated with MSCs.

population demonstrated typical MSC fibroblastic morphology, whereas the bead-positive population consisted of small, spherical cells consistent with lymphohematopoietic cells (Figure 1A and 1B). Fluorescence-activated cell sorter (FACS) analysis demonstrated that the bead-negative cells did not express the surface markers CD31, CD34, CD45, and CD117 but did express high levels of CD44 (95±0.6%), CD90 (99.1±0.1%), and CD105 (89±2.1%), in agreement with previous published data on MSC cell surface markers (Figure 1C).[7]

Murine Endothelial and Smooth Muscle Cell Harvest

To isolate mouse aortic endothelial cells (MAECs), thoracic aortas (n=10) were cut into 1- to 2-mm rings after adventitial removal and incubated with 0.25% trypsin for 20 minutes. Floating cells were harvested and cultured in medium-199 supplemented with 10% FBS (M-10). Cells were uniformly positive for factor VIII. Smooth muscle cells (SMCs) were isolated with the use of a modification of a previously described protocol.[8] Briefly, after MAECs were collected as described above, collagenase in Hanks' balanced salt solution (1 mg/mL) was added and incubated in 37°C for up to 3 hours with gentle agitation every 15 to 30 minutes. Floating cells were harvested, washed, and resuspended in M-10. Cells stained uniformly for smooth muscle actin. Passages 3 to 8 were used for experiments.

Conditioned Media Collection and Analysis

For enzyme-linked immunosorbent assay (ELISA), media were collected from plates of MAECs or MSCs (MSC conditioned media [MSC-CM]) after 24 hours of culture and analyzed by sandwich ELISA kits (vascular endothelial growth factor [VEGF], basic fibroblast growth factor [bFGF], placental growth factor [MCP-1], and placental growth factor [PlGF]) according to the manufacturer's directions. As a control, basal media were also analyzed. ELISA values were corrected for total cell protein.

Endothelial Cell and SMC Proliferation Assay

MAECs or SMCs (1×10^4 per well) were cultured in varying dilutions of MSC-CM or control wells of DM-10. When indicated, blocking antibodies to VEGF (10 μg/mL, Sigma) and/or blocking antibodies to bFGF (5 μg/mL, Sigma) were added to the MSC-CM. Cultures were continued for 72 hours, after which the cells were recovered and counted with a Coulter counter. Data are reported as the mean percent change in proliferation compared with control.

Animal Surgery and Cell Delivery

All animal procedures were approved by the institutional animal care and use committee. Under sedation, 12-week-old Balb/C mice (Jackson Laboratories, Bar Harbor, Me) underwent distal femoral artery ligation to create unilateral hindlimb ischemia. In preliminary studies, MSC injection immediately after femoral ligation failed to improve flow recovery. Therefore, in the present study, MSC injection was delayed by 24 hours to allow the mice to recover from surgery. MSCs (1×10^6 cells in 250 μL volume; n=10), MAECs (1×10^6 cells in 250 μL volume; n=8), or media alone (n=10) were injected in 6 sites in the right adductor muscle adjacent to and within 1 mm proximal or distal to the ligation site.

In Vivo Assessment of Limb Perfusion, Function, and Ischemic Damage

Blood flow recovery between mid-calf and mid-foot regions was measured with laser-Doppler perfusion imaging (LDPI) (Moor Instruments). For consistent measurements, imaging was performed after limb hair removal and after heating to 37°C to minimize temperature variation. Calculated perfusion is expressed as a ratio of the ischemic to normal limb.[9] Previous data suggest a close linear relationship between recovery of LDPI perfusion and remodeling of adductor collateral vessels.[10] A semiquantitative functional assessment of the ischemic limb was performed by a blinded observer using a modification of a clinical score (0=toe flexion, 1=foot flexion, 2=no dragging but no plantar flexion, 3=foot dragging).[11] Ischemic damage was also scored (0=no change, 1=mild discoloration, 2=moderate/severe discoloration, 3=necrosis, 4=amputation).

Western Blotting

Muscle samples were harvested 24 hours (for hypoxia-inducible factor-1α [HIF-1α] assay) or 7 days (for VEGF and bFGF assays) after surgery. Proteins were separated with the use of SDS-PAGE gels (10%) and incubated with antibodies to HIF-1α (1:800, Santa Cruz), VEGF (1:1000, Chemicon), bFGF (1:1000, Santa Cruz), or α-tubulin (1:2000, Santa Cruz). Relative quantification of proteins was determined with the use of Imagegauge software (Fuji Photo Film Co).

MSC Labeling and Tracking

Preliminary studies determined that 99% of MSCs were transduced with an adenovirus containing a reporter transgene at a multiplicity of infection (MOI) of 150 (data not shown). To track protein expression, cells were incubated with Ad.GFP or Ad.β-galactosidase at MOI of 150 for 2 hours and immediately injected into the adductor muscle (24 hours after surgery). To follow the fate of injected green fluorescent protein (GFP)+/MSCs, sections of adductor and calf muscle were examined with the use of a Nikon inverted fluorescent microscope. To follow the fate of β-gal+/MSCs, sections were developed with an X-gal kit (Invitrogen). For demonstration of vessels, adductor muscle sections were stained with a goat anti-mouse platelet–endothelial cell adhesion molecule (PECAM) antibody (Santa Cruz).

Immunofluorescence and Histological Analysis

For colocalization of MSCs and VEGF, MSCs were incubated ex vivo with carboxyfluorescein diacetate succinimidyl esters (CFSE) (Molecular Probes) and injected into adductor tissue. At day 7, cryostat sections of adductor muscle were fixed in methanol, blocked with 10% FBS for 30 minutes, and incubated with goat anti-mouse VEGF antibody (1:500, Santa Cruz) and then with phycoerythrin-coupled anti-goat antibody (Santa Cruz). Adductor muscle sections were stained with van Gieson's solution, and conductance arteries, identified by the presence of a continuous internal elastic laminae and muscle spindles, were counted. Total cross-sectional area was calculated with the use of Image-Pro software, with the smallest internal luminal distance measured as the radius. For collagen analysis, calf muscle sections were fixed in formalin and stained with 0.1% Sirius red. Collagen volume fraction was determined by measuring the percentage of the total area of stained tissue within a given field.

Statistical Analysis

All results are presented as mean±SEM. Statistical significance was evaluated with an unpaired Student *t* test for comparison between 2 groups or with ANOVA for comparison and contrast between multiple groups. A probability value of <0.05 was considered significant.

Results

MSC-CM Cytokine Content and Effect on Cell Proliferation

Previous studies demonstrated the importance of VEGF and bFGF in mediating tissue responses to ischemia.[12] Therefore, to characterize MSC release of these and other cytokines, we collected MSC-CM for 24 hours. With the use of ELISA, the release of VEGF (375 pg/μg protein from MSC-CM versus 34 pg/μg protein from MAECs; $P<0.01$), bFGF (2320 versus 25 pg/μg; $P<0.001$), PlGF (119 pg/μg versus nondetectable; $P<0.05$), and MCP-1 (150 versus 4 pg/μg; $P<0.05$) in MSC-CM was demonstrated (Figure 2A). Cytokines were not detected in basal culture media.

To examine whether MSC-CM exhibited biological effects, a series of endothelial cell and SMC proliferation assays were performed. MSC-CM significantly enhanced endothelial cell proliferation over control in a dose-responsive manner (Figure 2B). Anti-VEGF antibody partially attenuated the effects of MSC-CM, although there was still a significant mitogenic effect over control (450% over control without antibody versus 190% over control with antibody; $P<0.001$; Figure 2B). In contrast, anti-bFGF antibody had a smaller effect on endothelial cell proliferation (450% without antibody versus 375% with antibody; $P<0.05$). Anti-VEGF antibody had little effect on SMC proliferation, although anti-bFGF antibody significantly impaired SMC proliferation (82% over control without antibody versus 31% with antibody; Figure 2C). Similar to endothelial cell proliferation, there remained a significant increase in cell number over control despite blocking VEGF and bFGF, implying the mitogenic influence of multiple MSC-CM cytokines.

Analysis of HIF-1α Induction In Vivo

HIF-1α protein level, a sensitive marker of ischemia, was measured to assess adductor muscle injection site ischemia. Quantitative analysis of the HIF Western blot showed a 30-fold increase in the calf HIF protein at day 1 and a 15-fold increase in the calf HIF protein at day 3 over control (Figure 3). There was no HIF protein demonstrated in the adductor samples. These data imply no or minimal ischemia at the injection site.

Hindlimb Blood Flow and Functional Recovery

All animals survived surgery and reached the 21-day end point. In mice receiving media or MAECs, flow returned to approximately 50% of the nonischemic limb by day 14, with no difference in flow recovery between the 2 groups. In contrast, in MSC-treated mice, there was a significant improvement in flow (Figure 4A), and a dose-response relationship was demonstrated (Figure 4B). In mice receiving MSCs, there was an increase in the number of mid-thigh arteries (6.6 versus 4.1 in those mice receiving media [$P<0.05$] versus 4.3 in those mice receiving MAECs [$P<0.05$]; Figure 4D). Similarly, total arterial cross-sectional area was increased in those mice compared with control (10 453 versus 6723 μm^2 [$P<0.05$] versus 5125 μm^2 [$P<0.05$], respectively).

Improved flow recovery led to improved hindlimb appearance and function. Mice receiving media or MAECs experi-

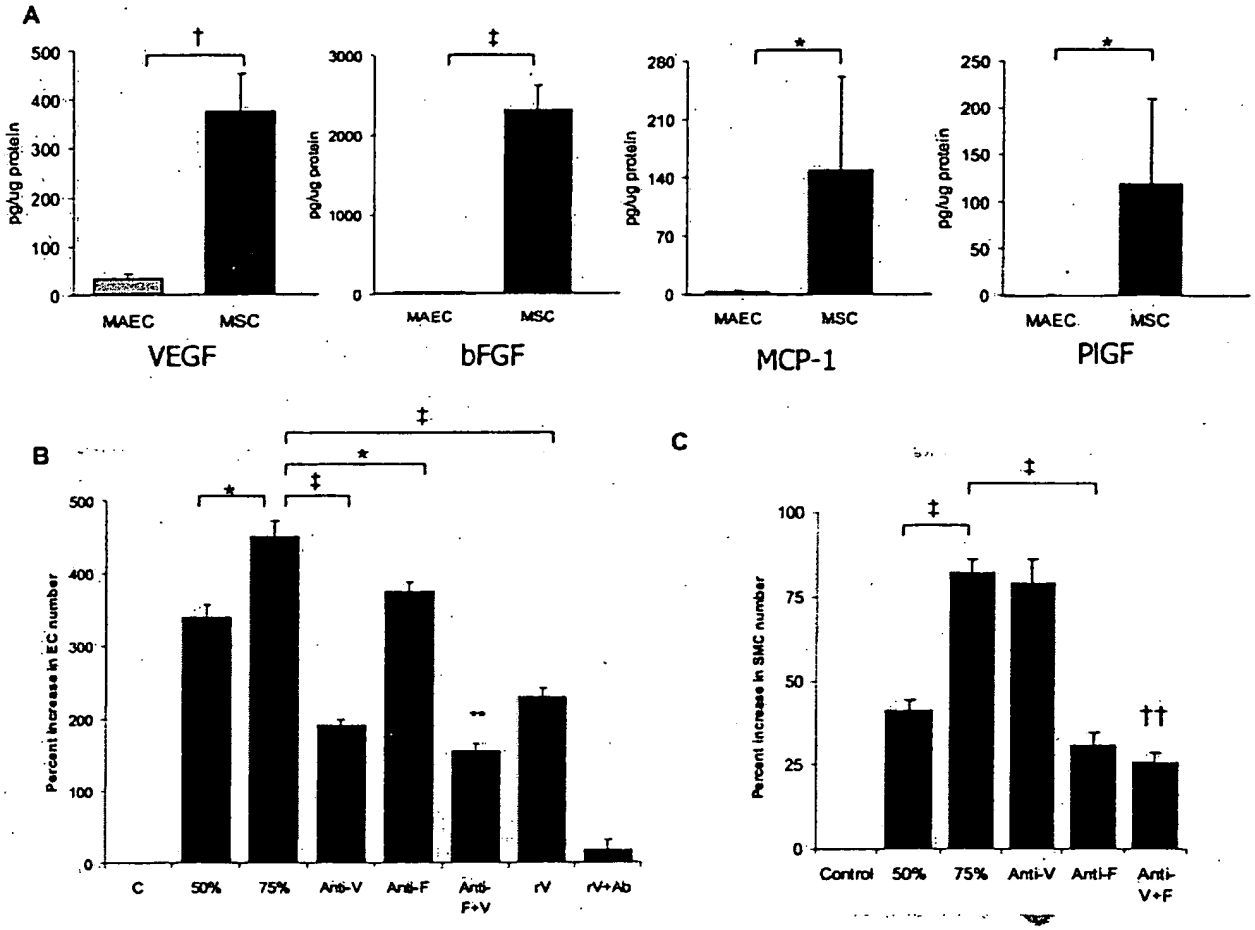

Figure 2. A, In vitro release of VEGF, bFGF, PlGF, and MCP-1 from MSCs and MAECs (*$P<0.05$, †$P<0.01$, ‡$P<0.001$). B, In vitro biological effects of 50% and 75% dilutions of MSC-CM on MAEC proliferation over control media with or without blocking VEGF (anti-V) and FGF (anti-F) antibodies (Ab). Also shown is rVEGF (rV) with or without anti-V as a control (*$P<0.05$, ‡$P<0.001$, **$P<0.0001$ over control). C, In vitro biological effects of 50% and 75% dilutions of MSC-CM on SMCs over control media with or without blocking antibodies (‡$P<0.001$, ††$P<0.05$ over control). EC indicates endothelial cells.

enced severe ischemic damage (ischemic score, 2±0.5 versus 2±0.62, respectively; P=NS), resulting in a 50% incidence of auto-amputation by day 21. However, mice receiving MSCs displayed less ischemic damage (ischemic score, 0.2±0.15; $P<0.05$ for both control group comparisons), with a 10% auto-amputation rate (Figure 5A). Similarly, in mice receiving media or MAECs, significant impairment of function remained at day 21 (1.75±0.24 in media group versus 2.4±0.3 in MAEC group; P=NS). However, in the MSC group, ambulatory impairment was less than both control groups (ischemic score, 0.4±0.18; Figure 5B).

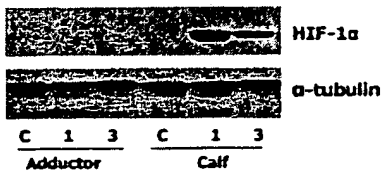

Figure 3. Strong HIF-1α protein induction in the calf muscle of the hindlimb in which the femoral artery was ligated, and absence of HIF-1α protein induction in the adductor muscle of the same limb. MSCs were injected into the adductor muscle.

Muscle Atrophy and Fibrosis

Significant calf muscle atrophy was noted in the media- and MAEC-treated mice (65.2±6.2% versus 60.3±6.5%, respectively; P=NS). MSC transplantation significantly attenuated this tissue loss (36.1±8.9%; $P<0.05$ versus media; Figure 5C). Muscle fibrosis was more pronounced in the media and MAEC groups compared with MSC (28.8±2.0% versus 29.2±2.0% versus 14.5±1.3%; $P<0.001$; Figure 5D). Fiber atrophy and disturbance of normal tissue architecture was also more evident in mice not receiving MSCs (Figure 5E).

MSC Tracking and Protein Expression in Adductor Muscle

To assess MSC viability and distribution, MSCs were transduced ex vivo with an Ad.GFP vector and injected immediately. The subsequent appearance of GFP+ cells implied viability and maintenance of the transcriptional and translational mechanisms. Few fluorescent cells were found in adductor sections taken at day 3. However, strongly fluorescent cells were observed in large numbers by day 7 and persisted through day 14 (Figure 6A, top). By day 21, cell

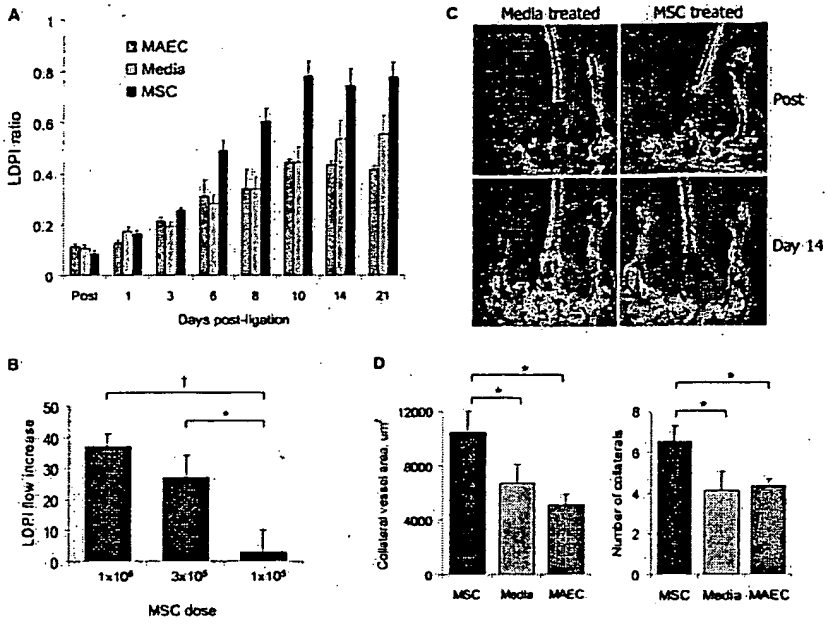

Figure 4. Perfusion analysis after cell treatment. A, LDPI expressed as a percentage of the normal limb. Flow recovery in MSC-treated animals was significantly better than that seen in control animals ($P<0.001$ for MSC trend vs both controls by ANOVA). B, Evidence of dose-response relationship. Percent increase in flow over media control at day 21 is shown (*$P<0.05$, †$P<0.01$). C, Representative LDPI images of flow recovery in a mouse receiving MSCs vs a mouse receiving media. Red is highest velocity; green, intermediate; and blue, lowest velocity. D, Number of mid-thigh collaterals (right) and total collateral cross-sectional area (left) in mice receiving MSCs compared with media or MAECs. *$P<0.05$.

numbers appeared to decline, and few cells were seen at day 28. GFP-positive cells were not seen in any calf muscle sections. With the use of β-gal labeling, MSCs were again found distributed widely between muscle fibers (Figure 6A, bottom). However, in conjunction with PECAM staining, multiple adductor muscle sections failed to demonstrate β-gal+ cells incorporated into vessels, suggesting in this model that MSCs did not transdifferentiate into endothelial cells or vascular SMCs.

Local Production of Arteriogenic Cytokines

To confirm that MSCs secreted arteriogenic cytokines in vivo, sections of adductor muscle were examined for colocalization of MSCs and VEGF. Clusters of CFSE+ cells were seen surrounding VEGF immunostaining (Figure 6B), suggesting local secretion of VEGF from the MSCs. Western blotting and ELISA confirmed significantly higher local adductor muscle production of bFGF and VEGF in those mice receiving MSCs compared with mice receiving media or MAECs (Figure 6C and 6D).

Discussion

To examine the potential role of paracrine mechanisms in augmenting collateral remodeling, we investigated the effects of MSCs because these cells play an important paracrine role in the marrow microenvironment. Previous studies have documented the release by MSCs of a wide array of cytokines that support hematopoietic stem and progenitor cell develop-

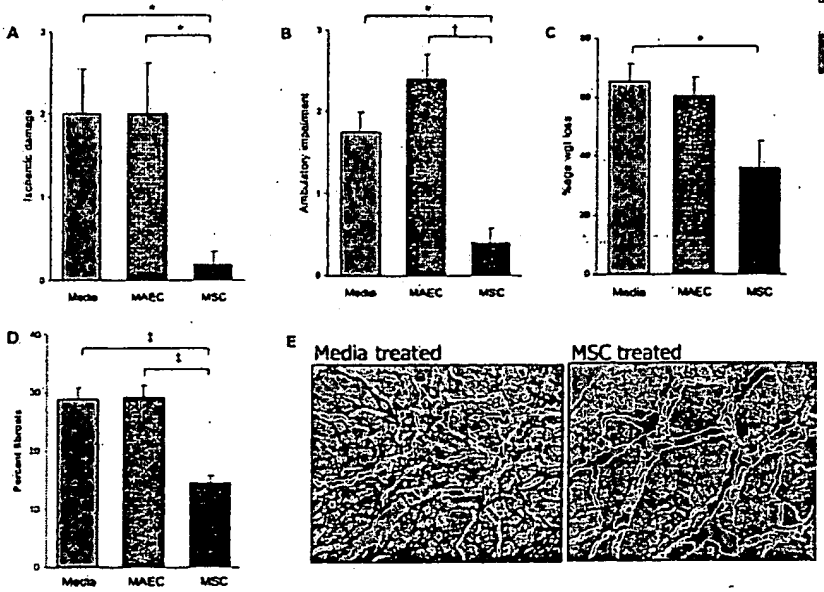

Figure 5. In vivo effects of MSC injection. A, Effects of MSC transplantation on ischemic damage (*$P<0.05$). B, Effects of MSC transplantation on ambulatory impairment (*$P<0.05$, †$P<0.01$). C, Effects of MSC transplantation on calf atrophy (*$P<0.05$). D, Direct injection of MSC into the adductor significantly reduces the degree of calf muscle fibrosis (‡$P<0.001$). E, Representative slides of calf muscle after treatment. Control demonstrates fiber atrophy and disarray (yellow) with heavy collagen staining (red). Treatment with MSCs preserves tissue architecture, with significantly less interstitial fibrosis.

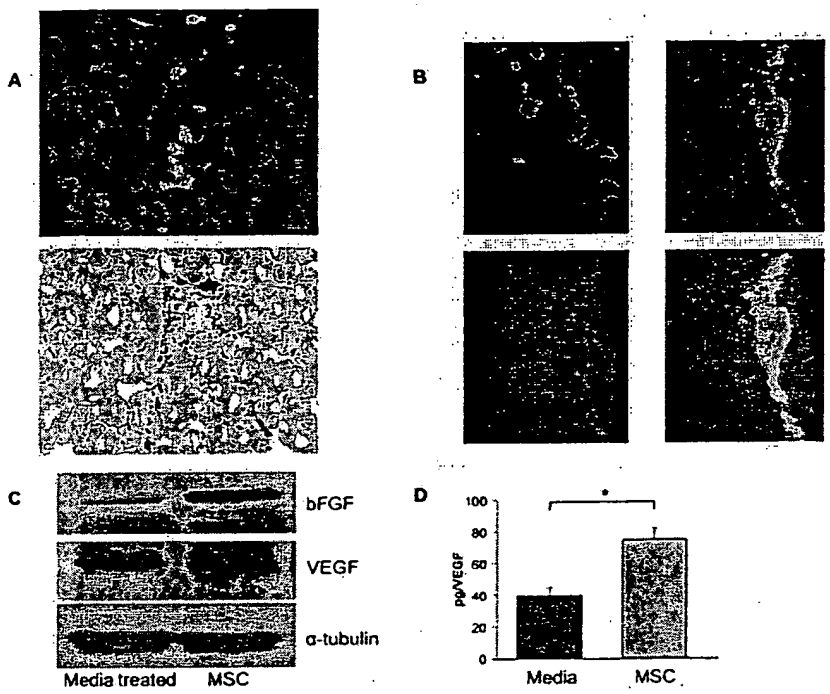

Figure 6. A, Top, ×60 magnification of adductor muscle sections 14 days after injection of GFP-labeled MSCs. 4′,6-Diamidine-2-phenylindole (DAPI) stains nuclei blue (in this section, mainly nuclei of skeletal muscle cells). Bottom, Several β-gal–positive MSCs were distributed between muscle fibers. B, Colocalization of CFSE-positive MSCs and VEGF. DAPI staining of nuclei (blue, top left), CFSE-labeled MSCs (green, top right), and VEGF staining (red, bottom left) are shown. CFSE-positive MSCs are surrounded by VEGF staining (mixed colors, bottom right), presumably reflecting secreted VEGF. C, Western blotting (day 7) demonstrates that MSC injection increases local production of VEGF and bFGF compared with control. D, ELISA confirms increased local production of VEGF after MSC injection (*$P<0.05$).

ment, as well as the secretion of other cytokines that are relevant to augmenting blood flow to ischemic tissue.[13]

In the present study, we demonstrate that MSCs secrete several important arteriogenic cytokines. MCP-1, for example, recruits monocytes to the perivascular compartment, where they orchestrate processes that ultimately lead to collateral vessel growth.[14] Subsequent SMC and endothelial cell proliferation are also crucial in this process, and while VEGF and bFGF enhance proliferation of endothelial cells, bFGF also enhances SMC proliferation. Furthermore, each of these cytokines has been used in a variety of animal models as a single agent to enhance the collateral response to ischemia.[15]

We found that injection of MSCs into the adductor muscles of the ischemic hindlimb significantly enhanced perfusion of ischemic tissue and collateral remodeling, lessened tissue damage, and improved limb function. These actions occurred without observable MSC incorporation into vessels. We also found that local production of bFGF and VEGF increased in MSC-injected tissue and documented colocalization of MSCs and VEGF. These results therefore demonstrate that stromal cells can augment collateral remodeling and appear to accomplish this mainly through paracrine pathways.

Previous studies have focused on stromal cell therapy regenerating myocardium when injected into an injured region.[5,16–18] Although these studies also documented increases in local capillary density, with MSCs found in capillary and arteriolar walls, to our knowledge the present study is the first to demonstrate the potential of stromal cells to augment collateral flow to ischemic tissue through paracrine mechanisms. Of interest, this effect was achieved with injection of the MSCs into tissue proximal to the site of arterial ligation and that manifested no or minimal ischemia.

Several explanations may account for the fact that whereas we demonstrated MSC-derived improvement in collateral function with no evidence of incorporation of stromal cells into mature collaterals, other studies have demonstrated incorporation of bone marrow–derived populations into blood vessels. First, in previous studies demonstrating incorporation of cells into vessels, cells were delivered into ischemic tissue, and incorporation was found to occur in capillaries. The local milieu is likely to be crucial in directing cells to differentiate, and injection into nonischemic tissue may not direct MSCs to incorporate into vessels and to differentiate into endothelial cells. Second, although such cells can incorporate into capillaries or small vessels present in injured, ischemic tissue, it is possible that they do not efficiently incorporate into remodeling collaterals. Third, although vessel wall incorporation of freshly isolated bone marrow mononuclear cells and endothelial progenitor cells has been documented, incorporation of MSCs is less well characterized. MSCs isolated in this study protocol may not represent a population able to differentiate and incorporate through an endothelial or smooth muscle lineage. If this is indeed the case, our data remain consistent with the concept that certain bone marrow–derived populations can enhance collateral remodeling without necessarily incorporating into the vessel wall. Finally, it is possible that small numbers of incorporated cells were simply missed during the section preparation. However, in light of the large increase in flow observed after local MSC injection, it seems unlikely that such a small number of cells could account for this effect.

The concept of paracrine effects mediating at least part of the effects of bone marrow cell therapy is not inconsistent with previous data. Several studies have demonstrated that freshly isolated bone marrow mononuclear cells contain mRNA for VEGF, bFGF, and angiopoietin-1, and local

increases in VEGF protein production after bone marrow mononuclear cell therapy were noted.[6,19–21] Endothelial progenitor cells were also found to release several cytokines, including VEGF and granulocyte-monocyte colony-stimulating factor.[22] Furthermore, injection of human-derived angioblasts into infarcted rat myocardium stimulated host endothelial cells to proliferate, suggesting that these angioblasts may also be a source of proangiogenic factors.[23] Therefore, these data combined with the present study imply that local cytokine release may be an important factor mediating the beneficial arteriogenic effects seen after delivery of bone marrow cells.

Local delivery of MSCs may also cause circulating stem/progenitor cells to home to the region of injury and contribute to healing. MSCs play an important hematopoietic supportive role and have an intimate relationship with stem/progenitor cells in the marrow cavity. In the present study we documented MSC release of several stem/progenitor cell chemokines, including VEGF and MCP-1. Previous studies have documented that MSCs release other stem/progenitor cell chemokines, including hepatocyte growth factor and stem cell–derived factor.[24] Therefore, it is highly likely that the collateral enhancing effects of cell therapy are mediated through multiple pathways, including paracrine effects on local vascular cells and chemoattractant effects leading to homing of circulating stem and or progenitor cells.

In addition to the direct therapeutic potential of these cells, the present study demonstrates that MSCs may be used as a vector for gene therapy. MSCs expressed adenoviral transgene product for at least 2 weeks after injection and, unlike fresh mononuclear cells, appeared relatively permissive to adenoviral transduction. This potential was previously exploited in a study demonstrating that MSCs engineered to overexpress interferon-β inhibited the growth of malignant cells in vivo.[25]

In summary, this study demonstrates that (1) MSCs produce a wide array of arteriogenic cytokines; (2) direct injection of MSCs into a region of forming collaterals improves perfusion and remodeling, lessens tissue damage, and enhances limb function in a mouse model of hindlimb ischemia; and (3) these effects appear to be mediated largely through paracrine mechanisms with local release of arteriogenic cytokines.

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Gene transfer into human bone marrow hematopoietic cells mediated by adenovirus vectors.

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Human bone marrow mononuclear cells (BMMNCs) and enriched CD34 positive (CD34+) cells were transduced with adenovirus vectors encoding Escherichia coli beta-galactosidase gene. Tranductions were carried out by 2· hour coincubation with adenovirus vectors at different multiplicities of infections (moi). Efficacy of gene transfer into BM cells and expression of th gene product (ie, beta-galactosidase) were studied using X-Gal histochemica staining and flow cytometric analysis. X-Gal staining demonstrated that the percentage of positive cells at mois of 5 to 500 was 3.4% to 34.5% for BMMNCs and 6.0% to 20.0% for enriched CD34+ cells. Similar results (1.5' to 35.7% for BMMNCs and 5.4% to 24.2% for enriched CD34+ cells) were obtained with flow cytometric analysis using fluorescein di-beta-D-galactopyranoside (FDG). Multicolor flow cytometry analysis, which include FDG, demonstrated that BM progenitors (CD34+ or CD34+CD38-), T cells (CD2+), B cells (CD19+), natural killer cells (CD56+), granulocytes, and monocytes all expressed the adenovirus transgene. To ascertain the effects of adenovirus vectors on normal BM progenitors, the numbers of colony formir unit-granulocyte/macrophage (CFU-GM), burst-forming unit-erythrocyte (BFU-E), and high-proliferative potential-colony-forming cells (HPP-CFC) after 24-hour coincubation with adenovirus vectors were determined. When BMMNCs or enriched CD34+ cells were incubated with adenovirus vectors : mois of 5 and 50, no significant differences in the numbers of CFU-GM, BFI E, and HPP-CFC were observed compared with the uninfected control cells.

However, the numbers of CFU-GM were significantly ($P < .01$) decreased w
BMMNCs or enriched CD34+ cells were incubated with adenovirus vectors
moi of 500, compared with the uninfected control cells. The adenovirus infec
cells, purified by cell sorting for FDG expression, were capable of growing i
culture and gave rise to various colonies (ie, CFU-GM, BFU-E, and HPP-CF
These data indicate that recombinant adenovirus vectors can be used to trans
genes to human BM hematopoietic cells with expression of the exogenous ge
at a high transduction efficiency.

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Transendocardial, Autologous Bone Marrow Cell Transplantation for Severe, Chronic Ischemic Heart Failure

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Background—This study evaluated the hypothesis that transendocardial injections of autologous mononuclear bone marrow cells in patients with end-stage ischemic heart disease could safely promote neovascularization and improve perfusion and myocardial contractility.

Methods and Results—Twenty-one patients were enrolled in this prospective, nonrandomized, open-label study (first 14 patients, treatment; last 7 patients, control). Baseline evaluations included complete clinical and laboratory evaluations, exercise stress (ramp treadmill), 2D Doppler echocardiogram, single-photon emission computed tomography perfusion scan, and 24-hour Holter monitoring. Bone marrow mononuclear cells were harvested, isolated, washed, and resuspended in saline for injection by NOGA catheter (15 injections of 0.2 cc). Electromechanical mapping was used to identify viable myocardium (unipolar voltage $\geq$6.9 mV) for treatment. Treated and control patients underwent 2-month noninvasive follow-up, and treated patients alone underwent a 4-month invasive follow-up according to standard protocols and with the same procedures used as at baseline. Patient population demographics and exercise test variables did not differ significantly between the treatment and control groups; only serum creatinine and brain natriuretic peptide levels varied in laboratory evaluations at follow-up, being relatively higher in control patients. At 2 months, there was a significant reduction in total reversible defect and improvement in global left ventricular function within the treatment group and between the treatment and control groups (P=0.02) on quantitative single-photon emission computed tomography analysis. At 4 months, there was improvement in ejection fraction from a baseline of 20% to 29% (P=0.003) and a reduction in end-systolic volume (P=0.03) in the treated patients. Electromechanical mapping revealed significant mechanical improvement of the injected segments (P<0.0005) at 4 months after treatment.

Conclusions—Thus, the present study demonstrates the relative safety of intramyocardial injections of bone marrow–derived stem cells in humans with severe heart failure and the potential for improving myocardial blood flow with associated enhancement of regional and global left ventricular function. (***Circulation.*** **2003;107:2294-2302.)**

Key Words: cells ■ heart failure ■ ischemia ■ revascularization ■ gene therapy

After myocardial infarction, chronically ischemic (hibernating) myocardium may persist in association with variable degrees of scar tissue. In most circumstances, native angiogenesis is insufficient to prevent the resultant remodeling when significant injury occurs. As a consequence, infarct-related heart failure remains a major cause of morbidity and mortality.

The understanding that vasculogenesis can occur in the adult has led to intense investigation into stem cell therapy. Several recent experimental studies have confirmed the potential of pluripotential cells in differentiating into cardiomyocytes and endothelial cells.[1,2] Further evidence from animal models has confirmed that pluripotential cells from

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bone marrow improve myocardial function and perfusion in the setting of ischemic heart disease.[3,4] In addition, recent publications[5,6] have described beneficial effects of intracoronary infusion of autologous, mononuclear bone marrow in the immediate postinfarction period in humans. A recent report by Tse et al[7] described improvement in myocardial perfusion and segmental contractility (as assessed by cardiac magnetic resonance imaging) in ischemic myocardial segments treated with catheter-based delivery.

The present study addresses primarily the safety of endocardial bone marrow mononuclear cell (BMMNC) injections and secondarily the hypothesis that endocardial injections of autologous BMMNCs (ABMMNCs) in patients with end-stage ischemic heart disease may promote neovascularization and may overcome the failure of the natural myocardial healing process.

Methods

Patient Population

This is a prospective, nonrandomized, open-label study of 21 patients with severe ischemic heart failure and no other option for standard revascularization therapies. Patients were enrolled sequentially, with the first 14 patients assigned to the treatment group and the last 7 patients to the control group. In accordance with the ethics committee's recommendations, an initial group of 4 patients was enrolled as a safety study. After 4 months' follow-up of the initially injected patients (once safety was determined), the remaining study patients were enrolled. All patients were placed on maximally tolerated medical therapy at time of enrollment. The following inclusion criteria were required for patient enrollment: (1) chronic coronary artery disease with reversible perfusion defect detectable by single-photon emission computed tomography (SPECT); (2) left ventricular (LV) ejection fraction (EF) <40%; (3) ineligibility for percutaneous or surgical revascularization, as assessed by coronary arteriography; and (4) signed, informed consent. Ineligibility for surgical or percutaneous revascularization procedures was determined by 2 expert committees: a surgical committee comprising 2 cardiovascular surgeons and a noninvasive cardiologist, and an interventional committee comprising 2 interventional cardiologists and 1 noninvasive cardiologist. Patients were not enrolled in the study if any 1 of the following exclusion criteria was met: (1) difficulty in obtaining vascular access for percutaneous procedures; (2) previous or current history of neoplasia or other comorbidity that could impact the patient's short-term survival; (3) significant ventricular dysrhythmias (sustained ventricular tachycardia); (4) LV aneurysm; (5) unexplained abnormal baseline laboratory abnormalities; (6) bone tissue with abnormal radiological aspect; (7) primary hematologic disease; (8) acute myocardial infarction within 3 months of enrollment in the study; (9) presence of intraventricular thrombus by 2D Doppler echocardiogram; (10) hemodynamic instability at the time of the procedure; (11) atrial fibrillation; or (12) any condition that, in the judgment of the investigator, would place the patient at undue risk.

The ethics committee of Pro-Cardiaco Hospital (Rio de Janeiro) and the Brazilian National Research Ethics Council approved the study protocol.

Baseline Evaluation

Baseline evaluation in the treatment group included a complete clinical evaluation (history and physical), laboratory evaluation (complete blood count, blood chemistry, C-reactive protein [CRP], brain natriuretic peptide [BNP], creatine kinase [CK]-MB and troponin serum levels), exercise stress test with ramp treadmill protocol,[8] 2D Doppler echocardiogram, dipyridamole SPECT perfusion scan, and 24-hour Holter monitoring.

The control group underwent the above-mentioned baseline evaluation except for 24-hour Holter monitoring, CK-MB, and troponin serum levels.

Periprocedural Evaluation

Patients in the treatment group had serum CRP, complete blood count, CK, troponin, and BNP (only 9 patients) levels measured and an ECG performed just before the procedure. Immediately after the procedure, another ECG and 2D Doppler echocardiogram were performed, and 24-hour Holter monitoring was begun. Serum CRP, CK, and troponin levels were also assessed at 24 hours. Patients were monitored in the cardiac intensive care unit for 48 hours after the injection procedure.

Bone Marrow Aspiration and Isolation of Mononuclear Cells

Approximately 4 hours before the cell injection procedure, bone marrow (50 mL) was aspirated under local anesthesia from the posterior iliac crest. BMMNCs were isolated by density gradient on Ficoll-Paque Plus (Amersham Biosciences). Mononuclear cells were exhaustively washed with heparinized saline containing 5% human serum albumin and filtered through 100-μm nylon mesh to remove cell aggregates. The cells were finally resuspended in saline with 5% human serum albumin for injection. A small fraction of the cell suspension was used for cell counting and viability testing with trypan blue exclusion. Cell viability was shown to be >90% (96.2±4.9%), assuring the quality of the cell suspension. Post-hoc characterization of leukocyte differentiation markers by flow cytometry and functional assays was done on another fraction of cells. The clonogenic capacity of hematopoietic progenitors was evaluated by colony-forming assays (granulocyte-macrophage colony-forming unit) as previously described.[9]

A high correlation between granulocyte-macrophage colony-forming units and $CD45^{lo}CD34^{+}$ cells was seen (Spearman $r=0.77$, $P=0.0012$). Fibroblast colony-forming assay was done as previously described[10] to determine the presence of putative progenitor mesenchymal lineages. Bacterial and fungal cultures of the clinically used cell preparations were performed and proved negative.

Antibodies and Staining Procedure for Fluorescence-Activated Cell Sorter Analysis

The following antibodies were either biotinylated or conjugated with fluorescein isothiocyanate (Pharmingen), phycoerythrin (PE), or PerCP: anti-CD45 as a pan-leukocyte marker (clone HI30), anti-CD34 as a hematopoietic progenitor marker (clone HPCA-II), anti-CD3 as a pan–T-cell marker (clone SK7), anti-CD4 as a T-cell subpopulation marker (clone SK3), and anti-CD8 as a T-cell subpopulation marker (clone SK1) from Becton Dickinson; anti-CD14 as a monocyte marker (clone TUK4), anti-CD19 as a pan–B-cell marker (clone SJ25-C1), and anti-CD56 as an NK-cell marker (clone NKI nbl-1), from Caltag Laboratories (Burlingame, Calif); and anti-HLA–DR (MHC-II, clone B8.12.2) from Beckman-Coulter. The biotinylated antibodies were revealed with Streptavidin-PECy7 (Caltag Laboratories). Three-color immunofluorescence analysis was used for the identification of leukocyte populations in total nucleated bone marrow cell suspensions. After staining, erythrocytes were lysed with the Becton Dickinson lysis buffer solution according to the manufacturer's instructions, and CD45 antibody was used to assess the percentages of leukocytes in each sample. Data acquisition and analyses were performed on a fluorescence-activated cell sorter Calibur with CellQuest 3.1 software (Becton Dickinson).

Transendocardial Delivery of ABMMNCs

In the cell-injection treatment group, patients were taken to the cardiac catheterization laboratory ≈1 hour before the anticipated arrival of the bone marrow cells from the laboratory. Left heart catheterization with biplane LV angiography was performed. Subsequently, electromechanical mapping (EMM) of the left ventricle was performed as previously described.[11] The general region for treatment was selected by matching the area identified as ischemic

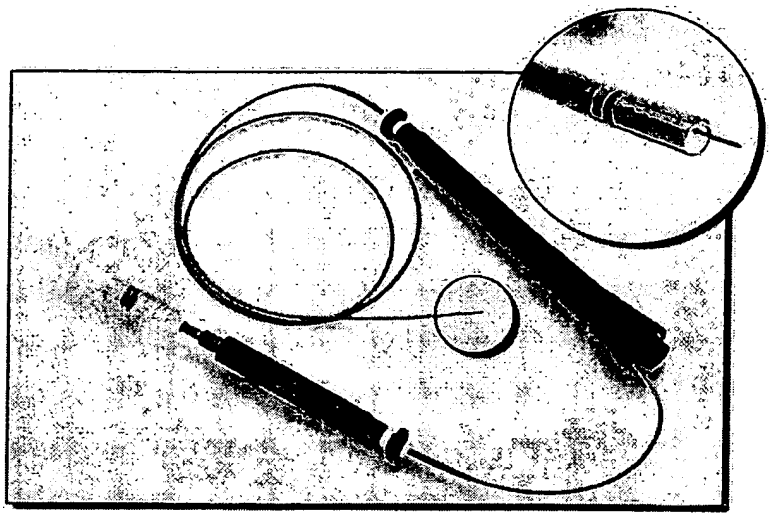

Figure 1. The NOGA Myostar injection catheter, with the needle in the extended position (insert).

by previous SPECT perfusion imaging. The electromechanical map was then used to target the specific treatment area by identifying viable myocardium (unipolar voltage ≥6.9 mV)[12] within that region. Areas associated with decreased mechanical activity (local linear shortening <12%, indicating hibernating myocardium) were preferred.

The NOGA injection catheter (Figure 1) was prepared by adjusting the needle extension at 0° and 90° flex and by placing 0.1 cc of ABMMNCs to fill the needle dead space. The injection catheter tip was placed across the aortic valve and into the target area, and each injection site was carefully evaluated before the cells were injected. Before every injection of cells into the LV wall, the following criteria had to be met: (1) perpendicular position of the catheter to the LV wall; (2) excellent loop stability (<4 mm); (3) underlying voltage >6.9 mV; and (4) presence of a premature ventricular contraction on extension of the needle into the myocardium. Fifteen injections of 0.2 cc (mean of $25.5 \pm 6.3 \times 10^6$ cells/patient) were delivered (Figure 2).

Two-Month Noninvasive Follow-Up Evaluation

All patients, both treated and control, underwent noninvasive follow-up evaluations at 2 months, which consisted of a clinical evaluation, ramp treadmill protocol, 2D Doppler echocardiogram, and dipyridamole SPECT perfusion scan. Patients in the treatment group had repeat 24-hour Holter monitoring. The ramp treadmill protocol was selected because it is better than standard incremental protocols in estimating functional capacity in these severely ill patients.[8]

The predicted $\dot{V}O_2$max was used to tailor the patient workload. Treadmill speed was initially 0.5 mph, and inclination was 0% to 10% with a planned duration of 10 minutes of exercise.[13,14] The echocardiographic data were analyzed by 2 independent, blinded, experienced observers. Images were stored digitally and analyzed offline. If a discrepancy between the readings of >5% was noted, a third blinded observer was called and a consensus achieved. The end-systolic volume (ESV), end-diastolic volume (EDV), and EF were measured according to standard protocols.

Dipyridamole stress and resting SPECT imaging were performed with the same stress procedure at baseline and at follow-up. Studies were read by a blinded, experienced observer. Approximately 740 MBq of technetium-99m sestamibi was injected at rest and after stress, with dipyridamole infusion at a rate of 142 μg/kg of body weight per minute infused for 4 minutes. One hour later, SPECT imaging was initiated, using a 15% window centered over the 140-keV photopeak. Acquisitions were performed with a 1-detector gamma camera (Ecam, Siemens), acquiring 32 projections over 180° (right anterior oblique 45° to left posterior oblique 45°) (low-energy, high-resolution collimation; 64×64 matrixes; and 35 seconds per projection). Short-axis and vertical and horizontal long-axis tomograms of the left ventricle were extracted from the reconstructed transaxial tomograms by performing coordinate transformation with appropriate interpolation. No attenuation or scatter correction was applied. Quantitative SPECT analysis was performed on an ICON workstation computer (Siemens). The analysis was performed with the use of a completely automated software package, with the exception of a quality-control check to verify the maximum count circumferential profiles. The methods for quantitative analysis have been previously described.[15,16] In brief, processing parameters, including the apical and most basal tomographic short-axis slices, the central axis of the LV chamber, and a limiting radius for myocardial count search, were automatically derived. Short-axis tomograms

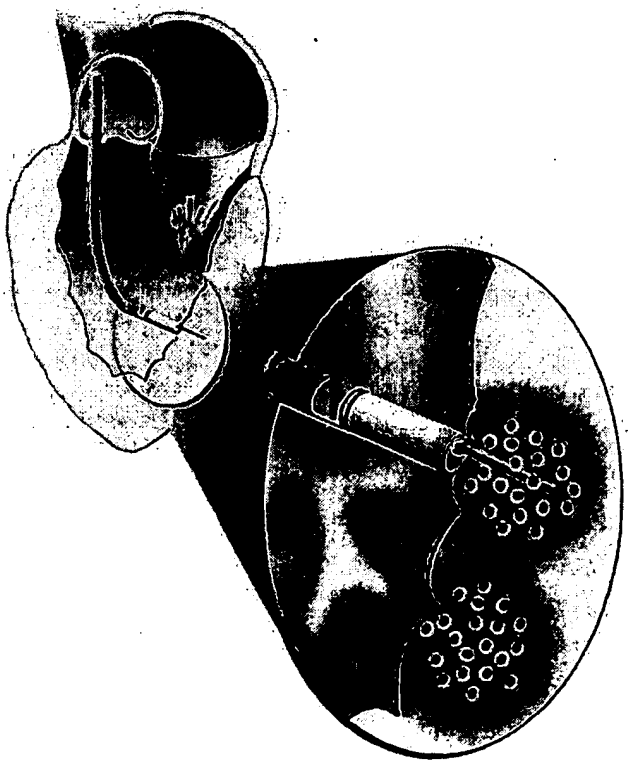

Figure 2. Injection catheter advanced into the left ventricle through the aortic valve. The catheter tip is placed against the endocardial surface (insert) with the needle extended into the myocardium delivering ABMMNCs.

TABLE 1. Demographics of the Treatment and Control Groups

	Treatment (n=14)	Control (n=7)	P
Age	56.9±9.8	64.3±7.2	0.1
Male gender, %	86	90	0.53
Hypertension, %	64	71	0.74
Diabetes, %	29	57	0.35
Hypercholesterolemia, %	79	57	0.35
Smoking, %	7	0	0.47
Previous myocardial infarction, %	100	100	1.0
Previous percutaneous coronary intervention, %	7	43	0.09
Previous coronary artery bypass grafting, %	64	86	0.61
Previous stroke, %	29	0	0.26
Peripheral vascular disease, %	57	71	0.66
Chronic renal failure, %	14	14	1.0
Multivessel disease, %	100	100	1.0

Values are mean±SD or percentage of patients.

were then sampled by using a maximum-count circumferential profile sampling technique with a cylindrical approach for sampling the body of the left ventricle and a spherical approach for sampling the LV apex. Comparisons were made to sex-matched normal limits.[16] Polar map displays and quantitative values were then generated to indicate stress myocardial perfusion defect extent and severity.[16,17]

Four-Month Invasive Follow-Up Evaluation

Patients in the control group did not undergo NOGA mapping or repeat LV angiograms at late follow-up (because of ethics committee recommendations).

Patients in the treatment group had 4-month invasive follow-up evaluations consisting of LV angiograms and EMM. LV angiography was performed through the femoral approach with the use of a 5F pigtail catheter. All angiograms were obtained in 2 planes—a 30° right anterior oblique view and a 60° left anterior oblique view—during a period of stable sinus rhythm. Ventricular volume was not measured during or after a premature beat. A 40-mm sphere was used as calibration device. LV EDV, ESV, and EF were calculated by 2 blinded, experienced observers who used the area-length method.[18]

EMM was performed according to established criteria[11] with a fill threshold of 15 mm. After the acquisition of points, postprocessing analysis was performed with a series of filters (moderate setting) to eliminate inner points, points that do not fit the standard stability criteria (location stability <4 mm, loop stability <6 mm, and cycle length variation <10%), points acquired during ST-segment elevation, and points not related to the left ventricle (eg, those in the atrium). A blinded, expert observer used a 12-segment bull's-eye to compare electromechanical values (unipolar voltage and local linear shortening) of injected segments at baseline and follow-up.

Statistical Analyses

Univariate differences in demographic characteristics (Table 1) between the control and treated groups were assessed with χ^2/Fisher's exact test and t tests for discrete and continuous variables, respectively. Multivariable logistic regression was also used to determine the independent relationship between each demographic variable and treatment group. No statistically significant differences between the 2 groups were found. Because each patient in both groups was used as his or her own control, changes between baseline and 8 weeks in the control and treated groups were assessed with paired t tests. Logistic regression analysis was utilized to compare medications (Table 2) at baseline, 8 weeks, and 16 weeks within the control and treatment groups and between the control and treatment groups.

TABLE 2. Percentage of Patients Receiving Selected Cardiac Medications at Baseline and 8- and 16-Week Follow-Up

	Baseline	8 Weeks	16 Weeks	P
ACE+ARB				
Control	86	86	86	1.0
Treatment	86	100	93	0.32
P	0.63*			
Nitrates				
Control	86	86	86	0.99
Treatment	93	93	93	0.91
P	0.95*			
β-Blockers				
Control	43	57	57	0.59
Treatment	71	71	64	0.73
P	0.94*			
Diuretics				
Control	71	71	71	1.0
Treatment	86	79	71	0.56
P	0.65*			
Ca channel blockers				
Control	14	14	29	0.49
Treatment	21	29	21	0.89
P	0.62*			

*P for comparison of all 3 time periods between treatment and control groups.

Comparisons of the changes from baseline to 8 weeks in the control and treatment groups were made with repeated-measures ANOVA. The ANOVA model included the control versus treatment and baseline versus 8 weeks as factors and also included the interaction between the 2 factors. A probability value <0.05 was considered statistically significant.

Results

Patient population demographics did not differ significantly between the treatment and control groups (Table 1). There were no significant differences in β-blocker, ACE inhibitor, or nitrate use between the 2 groups (Table 2).

Procedural Data

The total procedural time for mapping and injection was 81±19 minutes. Electromechanical maps comprised an average of 92±16 points. Patients received an average of 15±2 cell injections in a mean of 2±0.7 segments (6 inferior, 14 lateral, 2 anterior, and 5 septal). Each injection of 2 million cells was delivered in a volume of 0.2 cc. The cell population comprised a mean of 2.44±1.33% $CD45^{lo}CD34^{+}$ cells (Table 3).

Safety Data

One patient in the control group died 2 weeks after enrollment in the study and was not included in the analysis. A patient in the treatment group died at 14 weeks, presumably of sudden cardiac death. This patient had onset of severe angina and was found to be in asystole by emergency medical personnel. The patient had persistent improvement in cardiac

TABLE 3. Characteristics of Bone Marrow Mononuclear Cells Injected Into the Myocardium*

Cell Population and Phenotype	Percent of Injected Cells	No. of Cells Injected, ($\times 10^3$)/mm^2
Hematopoietic progenitor cells ($CD45^{lo}CD34^+$)	2.4±1.3*	57.4±61.4*
Early hematopoietic progenitor cells ($CD45^{lo}CD34^+HLA\text{-}DR^-$)	0.1±0.1	2.1±1.8
$CD4^+$ T cells ($CD45^+CD3^+CD4^+$)	28.4±10.8	537.0±265.7
$CD8^+$ T cells ($CD45^+CD3^+CD8^+$)	14.9±5.9	311.0±221.6
B cells ($CD45^+CD19^+$)	1.9±1.0	232.5±174.8
Monocytes ($CD45^+CD14^+$)	10.0±4.0	202.8±161.0
NK cells ($CD45^+CD56^+$)	1.2±0.5	21.2±13.5
Functional assay	**No. Colonies/10^6 BMMNC**	**No. of Cells Injected, ($\times 10^3$)/mm^2**
Fibroblast colony-forming assay	7.8±9.7	0.2±0.2
Granulocyte-macrophage colony-forming unit assay	719.6±385.3	16.4±18.5

Values are average±SD.

*Results for 14 patients in the treatment group, except: $CD34^+CD45^{lo}HLA\text{-}DR^-$, 13 patients; $CD45^+CD19^+$, 13 patients; $CD45^+CD14^+$, 11 patients; and $CD45^+CD56^+$, 9 patients.

function, as assessed by echocardiography. Baseline EF was 30% by echocardiography and increased to 57% at 2-month follow-up, demonstrating a similar response as the rest of the treatment group with regard to increased contractile function. In both cases, the families refused postmortem exams.

There were no major periprocedural complications. One patient had a transient episode of pulmonary edema that was easily reversed with loop diuretics after the procedure. No sustained arrhythmias were associated with the injection procedures, nor did any significant arrhythmias occur while the patients were hospitalized. There were no sustained ventricular arrhythmias found on 24-hour Holter monitoring at baseline or when repeated after the injection procedure and no significant differences in the number or percentage of premature ventricular contractions. No postprocedural pericardial effusions were seen on 2D Doppler echocardiograms. All patients were discharged on the third hospital day as per protocol.

Two-Month Noninvasive Follow-Up Evaluations

Of all baseline and follow-up laboratory values (Table 4), only serum creatinine and BNP levels varied between the control and treatment groups at follow-up. Follow-up serum creatinine levels were significantly elevated in the control group as compared with the treatment group ($P=0.03$). The levels of CRP at baseline and follow-up were not significantly different between the two groups (Table 4). There was a trend toward increased difference of BNP levels at follow-up between the two groups, with higher levels in the control group ($P=0.06$).

Patients in the treatment group experienced less heart failure and fewer anginal symptoms at the 2-month follow-up when compared with the control group, by both New York Heart Association (NYHA) and Canadian Cardiovascular Society Angina Score (CCSAS) distribution (Table 5). Baseline exercise test variables (METs and $\dot{V}O_2max$) were similar for the 2 groups. There was a significant increase, however, in METs and $\dot{V}O_2max$ at follow-up in the treatment group ($P=0.0085$ and 0.01, respectively). There was a trend toward improvement when these variables were compared with the control group ($P=0.08$ for both variables).

Baseline comparison of ESV, EDV, and LVEF between the treatment and control groups revealed significant differ-

TABLE 4. Laboratory Values for the Treatment and the Control Groups

	Treatment (n=14)	Control (n=7)	P
White blood cells, nL			
Before treatment	8.3±2.8	8.6±1.5	0.39
After treatment	8.3±2.1	9.2±1.4	0.19
P	0.85	0.14	
Creatinine, mg/dL			
Before treatment	1.17±0.32	1.35±1.02	0.60
After treatment*	1.10±0.26	1.63±0.08	0.030
P	0.23	0.09	
CRP, mg/dL			
Before treatment	1.00±0.70	0.76±0.50	0.43
After treatment	1.03±1.0	0.61±0.57	0.33
P	0.94	0.59	
BNP, pg/mL			
Before treatment	328.1±410.7	404.4±421.6	0.73
After treatment	281.8±286.6	565.1±366.3	0.06
P	0.91	0.19	
CK-MB, ng/mL			
Before treatment	2.67±0.42	NA	NA
24 Hours	3.08±1.43	NA	NA
P	0.35	NA	NA
Troponin, ng/mL			
Before treatment	0.14±0.09	NA	NA
24 Hours	1.13±0.84	NA	NA
P	0.0007	NA	NA

CK-MB indicates myocardial muscle creatine kinase isoenzyme; NA, not applicable.

*After treatment=2 months.

TABLE 5. Comparison of Baseline and 2-Month Follow-Up Values for the Treatment and Control Groups

	Treatment (n=14)	Control (n=7)	P*
NYHA class			
Before treatment	2.21±0.89	2.71±0.75	
After treatment	1.14±0.36	2.71±0.76	0.0001
P	0.0003	1.0	
CCSAS class			
Before treatment	2.64±0.84	2.57±0.97	
After treatment	1.28±0.61	2.14±0.89	
P	0.0001	0.06	0.001
Ramp treadmill			
METs			
Before treatment	5.09±2.5	5.07±1.96	
After treatment	6.68±2.35	5.16±2.45	0.078
P	0.0085	0.84	
$\dot{V}o_2$max			
Before treatment	17.96±8.78	17.75±6.85	
After treatment	23.38±8.31	18.08±8.58	0.08
P	0.01	0.84	
Echocardiogram			
ESV, cc			
Before treatment	146.78±53.46	89.42±26.23	
After treatment	123.21±47.88	98.85±20.52	0.041
P	0.026	0.36	
EDV, cc			
Before treatment	211.35±76.89	135.71±26.08	
After treatment	189.14±67.54	145±27.62	0.09
P	0.065	0.50	
EF, %			
Before treatment	30±5.56	36±11.73	
After treatment	35.5±7.85	31.85±7.55	0.029
P	0.027	0.31	
SPECT			
Total reversible defect, %			
Before treatment	15.15±14.99	10.71±16.60	
After treatment	4.53±10.61	32.28±37.25	0.022
P	0.016	0.23	
% Rest defect (50%)			
Before treatment	40.77±11.13	35.85±10.09	
After treatment	38.84±8.79	36.42±12.08	0.65
P	0.44	0.77	

*P values reflect comparison of the differences between treatment and control groups over time (see Methods).

ences: The control group had smaller LV volumes ($P<0.001$) and a trend ($P=0.054$) toward higher baseline EF. Cardiac function (measured by EF on echocardiograms) had an absolute increase of 6% over the 2-month follow-up period in the cell-treated group. In contrast, the mean EF decreased, although not significantly, in the control group. In addition, when the 2 groups were compared, the treatment group showed a significant improvement in EF after 2 months ($P=0.03$). Cardiac geometry, as assessed by ESV, also improved. A significant fall in ESV ($P=0.03$) and a trend toward reduction in EDV ($P=0.07$) were noted in the treatment group. Volumes remained unchanged within the control group. When the two groups were compared at follow-up, a significant reduction in ESV was seen in the treated patients ($P=0.04$).

Nuclear perfusion imaging studies were similar at baseline for the amount of total reversible defect and percent of rest defect with 50% activity (scar). Within the control group, there was no significant change in these two variables at follow-up. Within the treatment group, there was no significant change in rest defect, with 50% activity at 2-month follow-up, but there was a significant 73% reduction in total reversible defect ($P=0.022$; from 15.15±14.99% to 4.53±10.61%). A typical example of resolution of inferolateral ischemia (baseline to follow-up) in a cell-treated patient is shown in Figure 3A.

Four-Month Invasive Follow-Up Evaluations

Results from LV angiography at baseline and 4-month follow-up are shown in Table 6. There was a sustained improvement in LVEF from baseline, an increase from 20% to 29% at 4 months (31% relative increase) ($P=0.0003$) in the treated patients. There was also a continued reduction in ESV ($P=0.03$) at 4 months. EDV remained unchanged ($P=0.1$). Control group patients did not have repeat LV angiograms.

On EMM, segmental analysis revealed a significant mechanical improvement of the injected segments ($P<0.0005$) (Table 6). Significant improvement in mechanical function at the injection site is illustrated by EMM in Figure 3B. Unipolar voltage values did not change from baseline to follow-up.

Discussion

The present study describes for the first time ABMMNC transplantation with the use of transendocardial injections in patients with severe LV dysfunction, end-stage ischemic heart disease, and no other option for treatment. The results of our study suggest that injection of ABMMNCs is safe and improves perfusion and myocardial contractility when viable areas of myocardium are targeted.

Wound healing is a multifaceted process that involves complex interactions between inflammatory cells, cytokines, and a number of extracellular matrix proteins, and the development of new capillaries. Because the normal reparative mechanisms seem to be overwhelmed when clinically significant myocardial injury occurs, a logical next step would be to amplify one part of this response artificially by applying stem cells locally in the setting of ischemia or infarction when a large amount of heart muscle has been injured.

In experimental animals, bone marrow–derived cells have been shown to regenerate areas of infarcted myocardium and coronary capillaries,[1] thus limiting functional impairment after myocardial infarction. Transendocardial injection of ABMMNCs has been shown to increase myocardial contractility and perfusion in swine.[4] Various cell lineages have been used to generate evidence that bone marrow stem cells

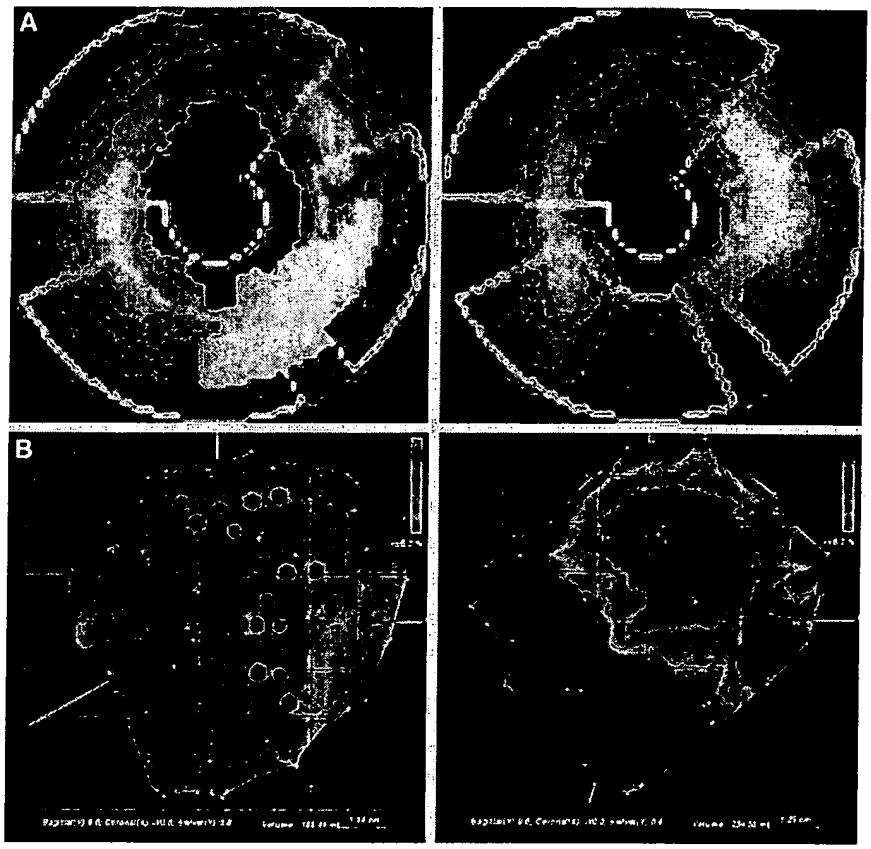

Figure 3. A, SPECT polar map at baseline, showing an area of inferolateral, reversible ischemia in white and nonreversible stress defect in black (left). Follow-up SPECT at 2 months, showing complete resolution of ischemic defect and basilar nonreversible defect with a decrease in nonreversible apical defect (right). B, Electromechanical maps from the same patient viewed from the inferior position. Mechanical map at the time of the injection procedure (left) shows the 15 injection sites in black distributed along the inferior wall. The follow-up mechanical map at 4 months (right) shows marked improvement in contractile function in the injected area.

differentiate into cardiomyocytes, endothelium, and smooth muscle cells.[19] Bone marrow hemangioblasts add to the development of new vessels, and mesenchymal stem cells can transdifferentiate into functional cardiomyocytes.[20] Recently, bone marrow–derived cardiomyocytes were demonstrated in hearts of women who received gender-mismatched bone marrow transplantation.[21] Moreover, bone marrow cellular components secrete a range of cytokines, fibroblast growth factor, and vascular endothelial growth factor,[22] which are involved in the natural process of angiogenesis. Endothelial progenitor cells have been implicated in neovascularization associated with postnatal vasculogenesis and are mobilized to peripheral circulation after acute ischemic events.[23]

In the present study, there is preliminary evidence that in humans, bone marrow–derived mononuclear cells are capable of enhancing perfusion, as shown by significant reductions in reversible stress defects on SPECT ($P=0.02$). Bone marrow–derived cells were purposefully injected into areas of hibernating myocardium. In hibernating areas, the underlying physiological state allows for restoration of myocardial function if myocardial perfusion is improved. We hypothesize that angiogenesis is the mechanism that allowed improvement in myocardial function in the patients in our study. Furthermore, we may speculate that an orchestrated sequence of events that includes not only the presence of the transplanted cells but also the action of cytokines and growth factors and intricate cell-to-cell interactions may all contribute to angiogenesis as an end result. Therefore, the resultant localized increase in contractility at cell injection sites, as seen by a significant increase in mechanical function on EMM, likely occurred as a consequence of an underlying improvement in perfusion. However, we cannot exclude the possibility that the injections themselves stimulated new blood vessel growth and enhanced function through the induction of angiogenic and important growth factors.

The homing process, which results in cell engraftment, may also play a key role in the success of cell therapy. After acute events, serum vascular endothelial growth factor levels rise significantly,[23] and it is likely that homing signals may be more intense in acute and subacute ischemic syndromes. In our patients, all of whom had chronic disease, we opted to perform transendocardial cell-therapy delivery because we believe that homing signaling may not be as intense and, therefore, might not be optimal for cell engraftment. It is also likely that a smaller number of cells is required to achieve the desired effect.

TABLE 6. Angiographic and EMM Results for the Treatment Group at 4 Months' Follow-Up (n=13)

	Before Treatment	After Treatment	*P*
LV angiogram			
EDV, cc	213.5±81.6	181±51.3	0.1
ESV, cc	174.1±78.7	133.5±54	0.03
EF, %	20±9	29±13	0.0003
EMM			
Unipolar voltage, mV	10.5±3.5	10.3±2.7	0.65
Local linear shortening, %	5.7±3.7	10.8±7.5	0.0005

EMM technology has been widely confirmed to be accurate for delineating and identifying scarred and viable myocardium and for differentiating degrees of infarct transmurality.[11,12,24] EMM thus offers a theoretical benefit over surgical or intracoronary approaches because viability of the site can be determined before each injection. Injections would then be performed only to targeted, viable areas of hibernating myocardium. Many treated sites targeted in this study were in areas of totally occluded epicardial vascular beds, making intracoronary delivery impossible. Furthermore, potential ischemia provoked by coronary manipulation is avoided. This approved procedure seemed safer for these chronically ill, high-risk patients because it avoided associated surgical morbidity and mortality.

Tse et al[7] recently demonstrated improvement in myocardial perfusion and segmental contractility after ABMMNC transendocardial injections. Those results are somewhat similar to results of the present study, although Tse and colleagues did not see improvement in global EF. The main difference between the studies is the significant baseline LV dysfunction present in our group (mean EF, 20%) as compared with a normal mean EF (56.9%) in the Tse study.[7] The preliminary data of Tse and colleagues also suggest the relative safety of the procedure.

The use of transendocardial delivery proved to be safe in our study, as cellular therapy was successfully delivered in every case without any major periprocedural events (eg, death, myocardial infarction, ventricular arrhythmias, cardiac perforation, pericardial effusion, or development of intramyocardial tumor). Troponin levels increased by a small but significant amount, consistent with delivery via intramuscular injection (Table 4), but the absolute rise was relatively small biologically. The stability between levels of CRP in the treatment and control groups suggests that we did not initiate a significant inflammatory reaction with cell injection.

The major limitations of this study are the small number of patients enrolled and the study design, which limits conclusions about efficacy. Because of ethics committee concerns, the control group was not enrolled concurrently with treated patients, did not receive a placebo injection, and did not undergo invasive follow-up. However, treatment and control groups had similar follow-up up to 2 months. The benefits seen in this study with cell therapy could be attributable to the placebo effect seen in phase 1 trials. Potential biases include selection bias (eg, tertiary hospital population) and investigator bias when assessing symptoms at follow-up (CCSAS and NYHA class) although echocardiographic, angiographic, and SPECT studies were read blindly. In addition, smaller LV volumes and a trend toward higher EFs were present in the control group. However, both groups were matched in terms of demographics, medication use, baseline laboratory values, functional status classification, treadmill workload, and $\dot{V}o_2max$. More importantly, similar baseline reversible and fixed ischemic defects were present in both groups, as one of the most important end points assessed in this study was the amount of reversible perfusion defect at follow-up. The end point of contractility is more difficult to evaluate in light of the differences between the groups at baseline; however, changes in opposite directions occurred at follow-up. In addition, the slightly better LVEFs and smaller hearts should logically have biased results against the cell-treated group.

Although the mechanisms by which cell therapy confers clinical benefit are not well understood, correlation between cell phenotype subpopulation analysis and long-term clinical outcomes is beyond the scope of the present study. Future analyses will be performed in this regard when longer-term follow-up is available.

The treatment of patients with heart failure has become increasingly important given the growing number of cases and their economic impact on the healthcare system.[25,26] More aggressive and widespread therapy in patients with chronic, ischemic heart failure will ultimately lead to a population harboring more advanced disease with a potential yearly mortality rate as high as 50%.[27] For these patients, therapeutic options remain limited. The very high-risk nature of the patient population represented in our study cohort is underscored by the fact that there was a death in both the control and the treatment groups. However, the significant improvement in LVEF noted in the treatment group on angiographic follow-up at 4 months (from 20% to 29%) may imply an improved clinical state and, it is hoped, provide some reduction in risks for the future.[28]

Conclusion

In this initial prospective, nonrandomized, open-label study in no-other-option coronary artery disease patients with LV dysfunction, we noted improvement in symptoms, cardiac function, and perfusion with transendocardial ABMMNC therapy, without any clinical evidence of significant harm from the procedure itself. We believe there may be clinical potential for this relatively novel therapy. Further investigation in a larger, randomized trial is warranted.

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Infarct Remodeling After Intracoronary Progenitor Cell Treatment in Patients With Acute Myocardial Infarction (TOPCARE-AMI)

Mechanistic Insights From Serial Contrast-Enhanced Magnetic Resonance Imaging

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Background—Experimental and initial clinical studies suggest that transplantation of circulating blood– (CPC) or bone marrow–derived (BMC) progenitor cells may beneficially affect postinfarction remodeling processes after acute myocardial infarction (AMI). To relate functional characteristics of the infused cells to quantitative measures of outcome at 4-month follow-up, we performed serial contrast-enhanced MRI and assessed the migratory capacity of the transplanted progenitor cells immediately before intracoronary infusion.

Methods and Results—In 28 patients with reperfused AMI receiving either BMCs or CPCs into the infarct artery 4.7±1.7 days after AMI, serial contrast-enhanced MRI performed initially and after 4 months revealed a significant increase in global ejection fraction (from 44±10% to 49±10%; $P=0.003$), a decrease in end-systolic volume (from 69±26 to 60±28 mL; $P=0.003$), and unchanged end-diastolic volumes (122±34 versus 117±37 mL; P=NS). Infarct size, measured as late enhancement (LE) volume, decreased significantly, from 46±32 to 37±28 mL ($P<0.05$). There was a significant correlation between the reduction in LE volume and global ejection fraction improvement. The migratory capacity of transplanted cells as assessed ex vivo toward a gradient of vascular endothelial growth factor for CPCs and stromal cell derived factor-1 for BMCs was closely correlated with the reduction of LE volume. By multivariate analysis, migratory capacity remained the most important independent predictor of infarct remodeling.

Conclusions—Analysis of serial contrast-enhanced MRI suggests that intracoronary infusion of adult progenitor cells in patients with AMI beneficially affects postinfarction remodeling processes. The migratory capacity of the infused cells is a major determinant of infarct remodeling, disclosing a causal effect of progenitor cell therapy on regeneration enhancement. (***Circulation***. **2003;108:2212-2218.**)

Key Words: cells ■ myocardial infarction ■ magnetic resonance imaging ■ remodeling

Myocardial salvage is the hallmark of successful reperfusion therapy, which has significantly reduced early mortality rates and improved prognosis in patients with acute myocardial infarction (AMI).[1] However, postinfarction heart failure resulting from ventricular remodeling processes remains a major challenge.[2] Recent experimental and initial clinical studies suggested that either intravenous infusion or intramyocardial injection of bone marrow–derived (BMC) or circulating blood–derived (CPC) progenitor cells may contribute to the regeneration of infarcted myocardium and enhance neovascularization of ischemic myocardium, resulting in sustained improvement of cardiac function.[3–12] In our previously published Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) pilot trial,[12] we demonstrated that intracoronary infusion of progenitor cells is associated not only with increased perfusion indices of infarcted segments but also with significant improvements in global and regional contractility and beneficial effects on postinfarction remodeling processes in patients with AMI. However, whether intracoronary infusion of progenitor cells contributes causally to the observed improvement in function remains enigmatic.

Contrast-enhanced MRI not only allows for a comprehensive quantitative analysis of the structural and functional

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consequences of myocardial injury but also is capable of distinguishing between reversible and irreversible dysfunction after AMI.[13–16] Thus, we performed serial contrast-enhanced MRI and assessed the migratory capacity of the transplanted progenitor cells immediately before intracoronary infusion into the infarct artery to relate functional characteristics of the transplanted progenitor cells to quantitative measures of outcome at 4-month follow-up.

Methods

Patients

Patients between 18 and 75 years of age were eligible for inclusion into the study if they had a first acute ST-elevation myocardial infarction that was treated acutely by coronary stenting with GP IIb/IIIa blockade. Exclusion criteria were the presence of cardiogenic shock (defined as systolic blood pressure <80 mm Hg requiring intravenous pressors or intra-aortic balloon counterpulsation); major bleeding requiring blood transfusion after acute reperfusion treatment; a history of leukopenia, thrombocytopenia, or hepatic or renal dysfunction; evidence of malignant diseases; or unwillingness to participate. The ethics review board of the Hospital of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. The angiographic, echocardiographic, PET, and coronary flow reserve data of 14 of the 28 patients have been reported previously.[12]

Study Protocol

The study protocol has been described previously.[12] In brief, patients were randomly assigned to receive intracoronary infusion of either BMCs or CPCs 4 days after AMI. In patients receiving BMCs, 50 mL of bone marrow aspirate was obtained in the morning of the day of cell transplantation. In patients receiving CPCs, 250 mL of venous blood was collected immediately after random assignment (24 hours after the AMI); mononuclear cells were purified and cultured ex vivo for 3 days and then reinfused into the infarct artery as described.[12,17–19] Cells were infused via an over-the-wire balloon catheter advanced into the stent previously implanted during the acute reperfusion procedure and inflated with low pressure to completely block blood flow for 3 minutes to allow for adhesion and potential transmigration of the infused cells through the endothelium. This maneuver was repeated 3 times to accommodate infusion of the total 10-mL progenitor cell suspension, interrupted by 3 minutes of reflow by deflating the balloon to minimize extensive ischemia. After completion of intracoronary cell transplantation, coronary angiography was repeated to ascertain vessel patency and unimpeded flow of contrast material.

Characterization of Infused Cells

The BMC suspension consisted of heterogeneous cell populations including hematopoietic progenitor cells, which were determined by fluorescence-activated cell sorter analysis using directly conjugated antibodies against anti-human CD34 (FITC; Becton Dickinson), anti-CD45 (Becton Dickinson), and CD133 (Miltenyi Biotech). Overall, a mean of $5.5\pm2.8\times10^6$ CD34/CD45-positive cells and $0.7\pm0.4\times10^6$ CD133-positive cells (in $238\pm79\times10^6$ mononuclear cells) were infused per patient. More than 90% of the CPC suspension (injected cells, mean $13\pm12\times10^6$) show endothelial characteristics, as demonstrated by Dil-acetylated LDL uptake and lectin binding and the expression of typical endothelial marker proteins, including vascular endothelial growth factor receptor (VEGFR2) (KDR) (Relia Tech), endoglin (CD105) (NeoMarkers), von Willebrand factor (Oncogene), and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Dianova).[12,17–19]

Assessment of Migratory Capacity of Transplanted Progenitor Cells

Immediately before intracoronary cell infusion, a sample of progenitor cells was resuspended in 500 μL endothelial basal medium (Cell Systems) and counted, and 2×10^4 CPCs or 1×10^6 BMCs were placed in the upper chamber of a modified Boyden chamber. Then, the chamber was placed in a 24-well culture dish containing endothelial basal medium and either 50 ng/mL VEGF for measuring migratory capacity of circulating CPCs or 100 ng/mL SDF-1 for measuring migratory capacity of BMCs. After 24 hours of incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with DAPI and were counted manually in 5 random microscopic fields by a blinded investigator.[19,20] Migrating BMCs were pelleted by centrifugation and were manually counted.

Magnetic Resonance Imaging

Cardiac MRI (1.5-T system; Magnetom Sonata, Siemens Medical Solutions) was performed 9 ± 4 days after myocardial infarction as well as 4 months after progenitor cell therapy. All images were acquired by use of a phased-array body surface coil with 4 to 12 elements during breath-holds (maximum, 12 seconds) and were ECG triggered. Cine images with a slice thickness of 8 mm were acquired throughout the entire left ventricle (LV) by use of contiguous 2D True-FISP (true fast imaging in steady-state precession) sequences. The typical in-plane resolution was 2.2×1.3 mm^2.

After intravenous application of Gd-DTPA (0.2 mmol/kg body wt), "late enhancement" (LE) imaging was performed with a delay time of 15 minutes. Contiguous inversion recovery 2D Turboflash (turbo fast low-angle shot) or 2D True-FISP sequences using an individually optimized inversion time of 170 to 280 ms were acquired. Again, the slice thickness was 8 mm; the in-plane resolution varied between 1.7×1.4 and 1.4×1.4 mm^2.

Data Analysis

Two patients with flow-limiting restenosis of the stented lesion in the infarct artery at follow-up angiography at 4 months were excluded from the analysis.

With the ARGUS software, LV function (ejection fraction, EF), end-systolic and end-diastolic volumes, LV mass normalized to body weight, and the volumes of the regions revealing LE were calculated from both examinations. In addition, regional EF was assessed by the same method restricted to slices with late hyperenhancement. Moreover, images were analyzed by use of a 17-segment model as recently proposed by the American Heart Association.[21] Segmental wall thickening was assessed semiquantitatively and judged visually to be either normal (2), hypokinetic (1), or akinetic (0) by 2 independent investigators (M.B.B., N.D.A.) blinded to the type of cells infused. The number of normokinetic, hypokinetic, and akinetic segments per patient was calculated and the wall motion score defined as the number of hypokinetic and akinetic segments per patient. Segmental functional recovery was defined as an increase from hypokinetic to normokinetic or an increase from akinetic to hypokinetic or normokinetic. Segmental LE extent was scored according to the following classification: 0%, >0 to ≤25%, >25 to ≤50%, >50% to ≤75%, and >75% of either volume extent or transmural extent. Furthermore, the amount of dysfunctional but viable segments (LE extent ≤25%) per patient was assessed.[11]

Statistical Analysis

Continuous variables are presented as mean±SD. Categorical variables were compared by the χ^2 test or Fisher's exact test. Statistical comparisons between initial and follow-up data were performed in a nonparametric fashion using the paired-sign test. Linear nonparametric correlation was calculated by the Spearman correlation. Multivariate analysis was performed using the linear regression model. Statistical significance was assumed if $P<0.05$. All statistical analysis was performed with SPSS software (version 11.0, SPSS Inc).

TABLE 1. Demographic, Clinical, and Angiographic Characteristics of the Study Population

	n=26
Age, y	51±9
Male sex, %	88
BMI, kg/m²	27±5
Hypertension, %	54
Hyperlipidemia, %	62
Diabetes, %	19
Smoking, %	65
Pack-y	29±18
Family history of CHD, %	35
CAD, 1-/2-/3-vessel disease, %	20/6/0
History of CAD, %	0
Infarct territory (anterior/inferior), %	50/50
Infarct-related vessel, %	
LAD	50
LCx	15
RCA	35
Time to revascularization, mean/median, h	23±30/13
Creatine kinase max, U/L	1381±1686
Creatine kinase–MB max, U/L	121±96
Type of progenitor cells: BMC/CPC, %	54/46
Time to MRI, d	9±4
Medication on discharge	
Aspirin, %	100
Clopidogrel, %	100
ACE inhibitor, %	96
β-Blocker, %	100
Statin, %	100

BMI indicates body mass index; CHD, coronary heart disease; CAD, coronary artery disease; LAD, left anterior descending coronary artery; LCx, left circumflex artery; and RCA, right coronary artery.

Results

The demographic, clinical, and angiographic data of the study population are summarized in Table 1. In all patients except 1 who experienced side effects from ACE-inhibitor therapy, aspirin, clopidogrel, statins, and β-blockers and ACE-inhibitor therapy were initiated during the hospitalization for AMI and continued until the 4-month follow-up examination.

Global LV Function

Figure 1 illustrates the data for the assessment of global LV function at the time of progenitor cell transplantation and at 4-month follow-up. Global LV EF increased significantly, from 44.1±9.9% (mean±SD) to 48.9±9.8% (Figure 1A), and end-systolic LV volume decreased significantly, from 69.4±25.5 to 59.5±28.1 mL (Figure 1C), whereas end-diastolic LV volume remained unchanged (121.6±33.7 versus 116.9±36.7 mL; Figure 1B). LV mass decreased slightly but significantly, from 84.6±15.6 to 78.6±15.1 g/m²; $P=0.04$; Figure 1D).

Regional LV Function

As illustrated in Figure 2, regional LV function was significantly improved at 4-month follow-up. Importantly, the number of akinetic segments per patient was reduced profoundly, from 2.7±1.9 at the time of cell therapy to 1.2±1.6 at 4-month follow-up ($P<0.001$), whereas the number of normokinetic segments increased significantly, from 9.9±2.9 to 12.3±2.8 ($P<0.001$).

Infarct Size and Functional Improvement

Infarct size as measured by the volume of LE varied within the patient population. Of the 23 patients with hyperenhancement on the scan at the time of cell therapy, 22 had hyperenhancement on the scan at 4-month follow-up, and all 22 had hyperenhancement in the same territories on both scans. Most importantly, LE volume decreased significantly, by ≈20%, from 46±32 mL at the time of cell therapy to 37±28 mL ($P<0.05$) at 4-month follow-up. Regional LV EF in slices with hyperenhancement increased significantly, from 43.2±11.4% to 47.6±11.5% ($P<0.005$). There was a close correlation between changes in global EF and regional EF within LE segments ($r=0.8$; $P<0.001$).

LE volume at the time of cell therapy did not correlate with future improvement in either global EF ($r=0.24$; $P=0.23$) or regional EF in slices with hyperenhancement ($r=0.16$; $P=0.47$). In contrast, however, as illustrated in Figure 3, there was a significant correlation between the reduction in LE volume and the improvement in global EF (Figure 3A) and in wall thickening (Figure 3B) 4 months after progenitor cell therapy.

Figure 4 shows the percentage of improved segments at 4-month follow-up as a function of LE at the time of cell therapy. Although initially dysfunctional segments without any infarction demonstrated the highest incidence of improvement at follow-up, neither the extent (Figure 4A) nor the transmurality (Figure 4B) of hyperenhancement predicted future functional recovery. For example, 48 of 65 segments (74%) without any infarction on the scan at the time of cell therapy improved on the scan at 4-month follow-up, but recovery rates were essentially identical, with 50% for >0% to ≤25% transmurality of LE, 47% for transmurality of LE >25% to ≤50%, 43% for transmurality of LE >50% to ≤75%, and 46% for LE transmurality >75%. Thus, almost 50% of dysfunctional segments with varying transmurality of infarction improved regardless of the initial extent of LE transmurality. Finally, although the extent of regional contractile dysfunction was significantly associated with the extent of LE initially ($r=0.54$; $P<0.005$) and at 4-month follow-up ($r=0.49$; $P=0.01$), the extent of LE at the time of cell therapy did not predict functional improvement of regional contractile function at 4-month follow-up ($r=-0.098$, $P=0.633$).

Table 2 summarizes the univariate predictors for global improvement in contractile function. The only statistically significant predictive variable was change in LE volume, whereas neither the initial LE volume nor the initially determined dysfunctional but viable region by MRI predicted the change in global LV EF at 4-month follow-up. As reported in our initial report,[12] there was no difference between CPCs and BMCs with respect to improvement of global LV function.

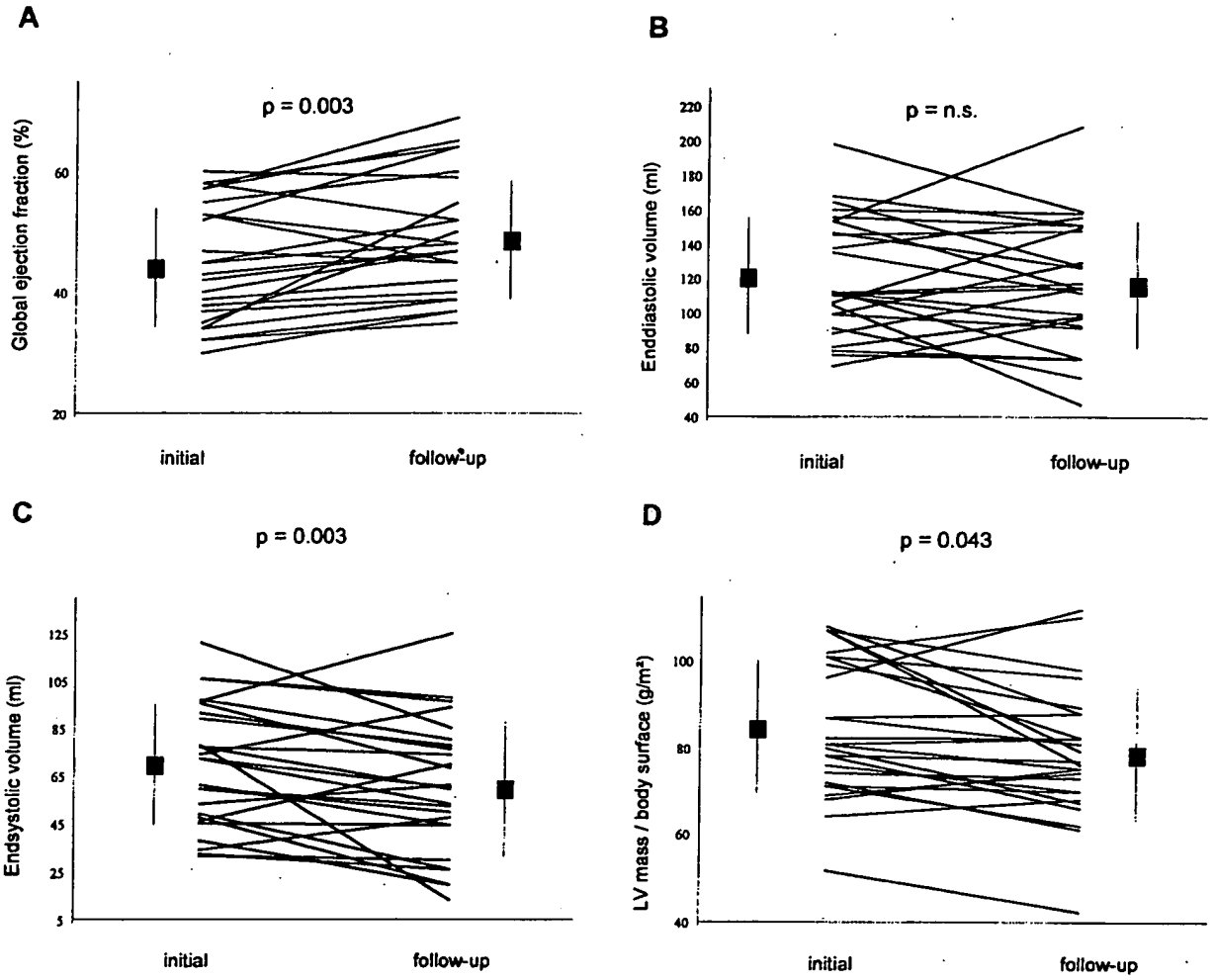

Figure 1. Comparison of initial and follow-up global EF (A), end-systolic volume (B), end-diastolic volume (C), and LV mass normalized for body surface (D). Error bars indicate mean±SD.

Number and Migratory Capacity of Transplanted Progenitor Cells and Infarct Remodeling

The absolute number of the infused progenitor cells did not correlate with improved global or regional LV function or with infarct size reduction when total cell numbers or subpopulations were used, eg, CD34/CD45- or CD34/CD133-positive cells (global EF: CPCs, $r=0.18$, $P=0.6$; BMCs, $r=-0.16$, $P=0.6$; infarct size: CPCs, $r=-0.16$, $P=0.6$; BMCs, $r=-0.004$, $P=1.00$). No significant differences were detected in functional improvement when cell numbers were dichotomized (data not shown).

The migratory capacity of the infused progenitor cells was assessed in 15 of the 26 patients. The VEGF-induced migratory capacity of CPCs ranged from 0.8 to 56 cells/high-power field (n=11; median, 11 cells/high-power field), and the SDF-1–induced migratory capacity of BMCs ranged from 13.5 to 102 (n=4; median, 51.5 cells/high-power field). Because different stimuli were used to assess migration of CPCs and BMCs, we dichotomized the migratory capacity. As illustrated in Figure 5, there was a close relation between migratory capacity and reduction of LE volume. Despite similar values of LE volume at baseline, the absolute reduction in LE was significantly greater in patients receiving cells with high migratory capacity than in those receiving cells with low migratory capacity (-12.5 ± 16 versus 9 ± 17 mL; $P<0.05$). Similar differences were also detected when only patients receiving CPCs were stratified ($P<0.05$).

To identify independent predictors of infarct remodeling after intracoronary progenitor cell infusion into the infarct artery in patients with AMI, we performed a multivariate analysis including all parameters that were statistically significant or approached statistical significance by univariate analysis or that are known to influence infarct size. As demonstrated in Table 3, the migratory capacity of the transplanted progenitor cells remained the strongest statistically significant independent predictor of infarct size reduction as measured by reduction of LE volume. The only other independent predictor was the baseline EF, whereas neither initial infarct size nor age, sex, or time to revascularization remained independent predictors. Thus, the migratory capacity of infused cells is a major independent determinant of infarct remodeling after progenitor cell therapy in patients with AMI.

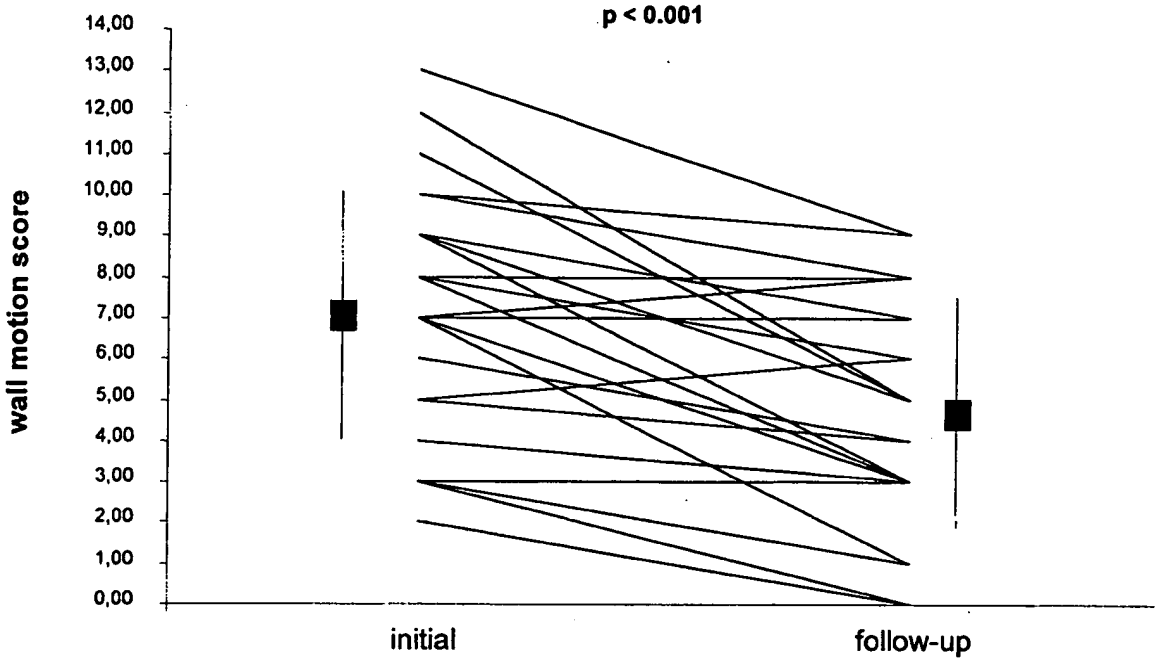

Figure 2. Wall motion score initially after cell therapy and at 4-month follow-up.

Discussion

The results of the present study extend our previously reported observation[12] that transplantation of adult progenitor cells is associated with significant beneficial effects on LV remodeling processes in patients with AMI. Serial contrast-enhanced MRI provided novel and unique insights into potential mechanisms involved in the observed functional improvement: Cell therapy was associated with a significant reduction in infarct size as measured by the volume of LE at 4-month follow-up, the reduction of LE volume correlated directly with the improvement of global LV EF, and both global and regional contractile recovery were independent of the initial LE volume.

Most importantly, however, the present study demonstrates that the functional capacity of the transplanted progenitor cells is a major independent determinant of subsequent infarct remodeling after intracoronary cell transplantation. Interestingly, the functional activity of the cells as assessed by their migratory activity was more informative than the cell number. This may be because the cell numbers infused were within a rather narrow range (75% of the patients received 4 to 18×10^6 CPCs or 150 to 300×10^6 BMCs). However, it is more likely that the functional activity at least in part can override differences in cell numbers. Taken together, these data for the first time suggest a causal relation between progenitor cell therapy and LV regeneration enhancement in patients with AMI.

In our initial report of the first 20 patients included in the TOPCARE-AMI trial,[12] we demonstrated that transplantation of adult progenitor cells was associated with a significant improvement in global LV EF and reduced end-systolic volumes as assessed by LV angiography. The present study now corroborates these findings by using a more robust and accurate method of assessing LV function, namely, MRI. Especially in the presence of a distorted LV geometry caused by previous myocardial infarction, MRI provides more reliable data because of its ability for 3D visualization of the LV cavity and LV wall. The only available study systematically

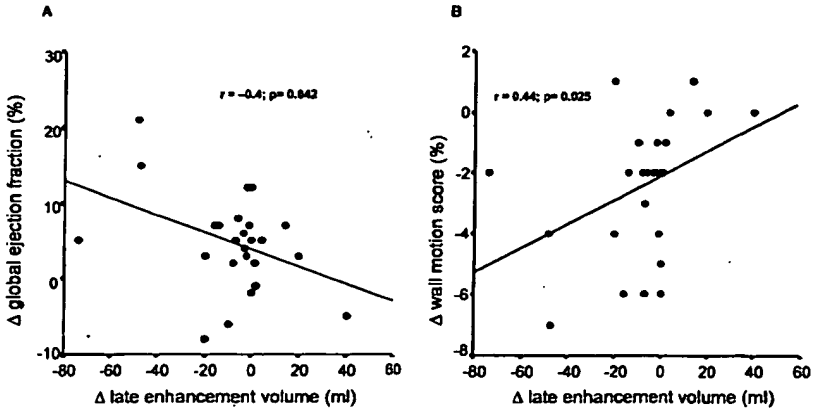

Figure 3. Correlation between changes in global EF (A) and wall motion score (B) and changes in LE volume (n=26).

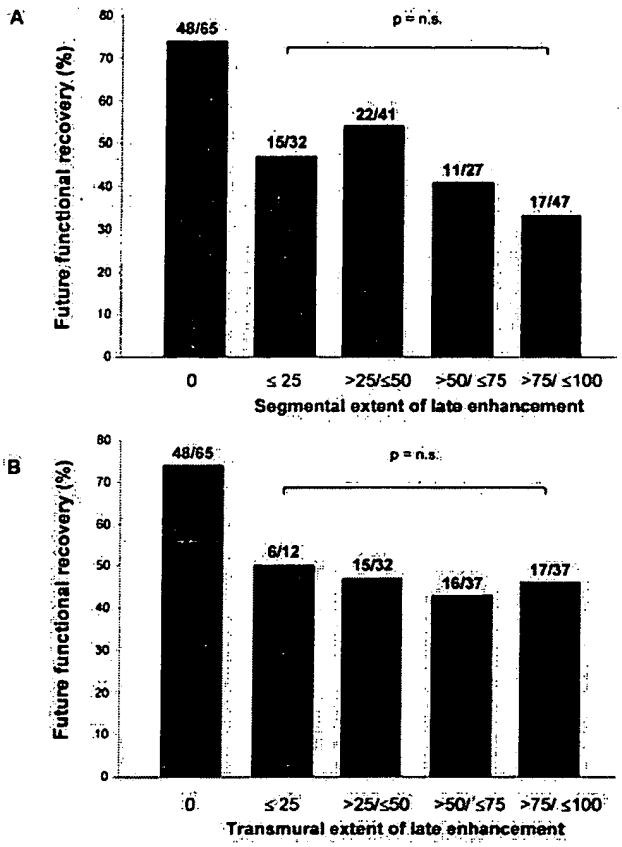

Figure 4. Percentage of improved segments as a function of regional LE extent and transmurality.

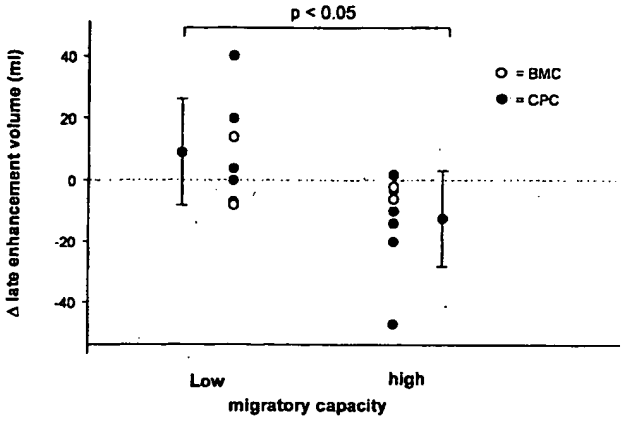

Figure 5. Migratory capacity of transplanted progenitor cells (dichotomized into high and low by use of median values) and infarct remodeling as measured by reduction in LE volume (n=15).

investigating LV remodeling by serial MRI at 5 days and 6 months after AMI treated either with percutaneous coronary intervention or thrombolysis revealed a significant increase in both end-systolic and end-diastolic LV volumes, with essentially unchanged LV EF.[22] Thus, preservation of LV EF occurred at the expense of increased LV volumes, indicating postinfarction remodeling processes. In contrast, in the present study, LV EF increased significantly but end-systolic LV volume decreased and end-diastolic volume remained unchanged over time, suggesting a beneficial effect of progenitor cell transplantation on LV remodeling processes.

Previous experimental studies suggested that the improvement in ventricular function after experimentally induced myocardial infarction is a result of stimulated neoangiogenesis preventing late myocardial remodeling through enhanced myocardial blood flow, rescue of hibernating myocardium, reduction of myocardial fibrosis, and decreased apoptosis of hypertrophied myocytes in the peri-infarct region.[4,5,23,24] In addition, Orlic et al[3] reported that intramyocardial injection of BMCs led to regeneration of significant amounts of contracting myocardium, suggesting that the de novo generation of myocardium may contribute to amelioration of the outcome of myocardial infarction after local delivery of adult progenitor cells. Indeed, we have recently demonstrated that CPCs retain the capability to transdifferentiate into functional cardiac myocytes.[25]

However, prerequisite for the success of cell therapy is the homing and, thus, engraftment of transplanted cells into the target area, especially if an intravascular route of administration is chosen. Therefore, we reasoned that the migratory capacity of adult progenitor cells toward their physiological chemoattractant might reflect their homing capacity into the infarcted area. Both VEGF and SDF-1 are profoundly upregulated in hypoxic tissue,[26–29] suggesting that VEGF and SDF-1 may constitute homing signals to recruit circulating progenitor cells to enhance endogenous repair mechanisms after critical ischemia. The results of the present study now demonstrate that the migratory capacity of transplanted progenitor cells is an independent predictor of infarct remodeling as measured by MRI-determined LE volume. Taken together,

TABLE 2. Univariate Analysis of Global EF Improvement

	ΔEF, *r*	*P*
Age, y	0.003	NS
Sex, M/F	0.07	NS
Creatine kinase–MB max	−0.157	NS
Time infarct to MRI	−0.129	NS
Time MRI to cell therapy	0.095	NS
Infarct territory (anterior/inferior)	0.25	NS
Type of progenitor cells: BMC/CPC	0.29	NS
Initial LE volume	0.24	NS
Dysfunctional but viable (MRI)	0.22	NS
Reduction of LE volume	−0.4	0.04

TABLE 3. Multivariate Analysis of Independent Predictors of Infarct Remodeling as Measured by Reduction in LE Volume

	ΔLE Volume Standardized Coefficient β	*P*
Sex	0.09	NS
Age	−0.11	NS
Time to revascularization	0.48	NS
Baseline EF	−0.53	0.01
Baseline LE volume	0.28	NS
Low/high migration	−0.738	0.004

the improvement in local contractile function associated with a reduction in infarct size being independently determined by the functional capacity of infused progenitor cells to migrate toward their physiological chemoattractants discloses a causal relationship between transplantation of progenitor cells and regeneration enhancement in patients with AMI.

Obviously, the present clinical study cannot disclose the cellular mechanisms associated with the improved LV contractile function after progenitor cell therapy. However, the results of the present study demonstrate that the intracoronary infusion of adult progenitor cells is associated with a profound reduction of infarct size, as measured by the volume of MRI-determined LE. This reduction in MRI-determined infarct size directly correlated with improved global and regional contractile LV function, suggesting that local contractile functional recovery is indeed beneficially affected by the infusion of progenitor cells into the infarct artery. Whereas previous studies have firmly established that the magnitude of long-term functional recovery is inversely related to the extent and transmurality of hyperenhancement,[14,15] local contractile recovery was entirely independent of both the initial extent and transmurality of irreversibly injured myocardium in our patients treated with intracoronary progenitor cell infusion. Instead, infarct remodeling as measured by the reduction in LE volume was independently predicted by the migratory capacity of the infused progenitor cells. These data indicate that cell therapy may beneficially modify the healing process of myocardial infarction. Given that the improvement of global LV function was predominantly a result of an improved contractility in LV slices with evidence for LE initially, the effects of progenitor cell therapy on postinfarction LV remodeling indeed appear to include rescue of "irreversibly" dysfunctional myocardium early after AMI. However, whether this novel form of regeneration enhancement therapy associated with augmented myocardial salvage will translate into sustained improvement in LV function and prognosis after AMI awaits the results of larger-scale randomized trials.

Acknowledgments

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1: Lancet. 2004 Jul 10;364(9429):141-8.

Comment in:

- Lancet. 2004 Jul 10;364(9429):121-2.

ELSEVIER SCIENCE FULL-TEXT ARTICLE

Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinic trial.

Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breide C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein Ganser A, Drexler H.

Department of Cardiology, Hanover Medical School, Hanover, Germany.

BACKGROUND: Emerging evidence suggests that stem cells and progenito derived from bone marrow can be used to improve cardiac function in patien acute myocardial infarction. In this randomised trial, we aimed to assess whe intracoronary transfer of autologous bone-marrow cells could improve globa ventricular ejection fraction (LVEF) at 6 months' follow-up. METHODS: Af successful percutaneous coronary intervention (PCI) for acute ST-segment elevation myocardial infarction, 60 patients were randomly assigned to eithe control group (n=30) that received optimum postinfarction medical treatmen bone-marrow-cell group (n=30) that received optimum medical treatment an intracoronary transfer of autologous bone-marrow cells 4.8 days (SD 1.3) aft Primary endpoint was global left-ventricular ejection fraction (LVEF) chang baseline to 6 months' follow-up, as determined by cardiac MRI. Image analy were done by two investigators blinded for treatment assignment. Analysis w protocol. FINDINGS: Global LVEF at baseline (determined 3.5 days [SD 1. PCI) was 51.3 (9.3%) in controls and 50.0 (10.0%) in the bone-marrow cell g (p=0.59). After 6 months, mean global LVEF had increased by 0.7 percentag points in the control group and 6.7 percentage points in the bone-marrow-cel group (p=0.0026). Transfer of bone-marrow cells enhanced left-ventricular s function primarily in myocardial segments adjacent to the infarcted area. Cel transfer did not increase the risk of adverse clinical events, in-stent restenosi proarrhythmic effects. INTERPRETATION: Intracoronary transfer of autolo bone-marrow-cells promotes improvement of left-ventricular systolic functio patients after acute myocardial infarction.

Publication Types: